Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary information

Disulphide-Mediated Site-Directed Modification of Proteins

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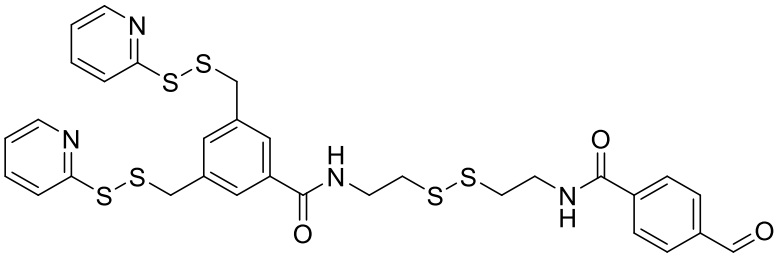
General methods and reagents

Reagents and purifications

Commercially available reagents were purchased from Sigma-Aldrich at the highest possible quality and used without further purification. DOTA-maleimide (cat# C105) and NOTA-maleimide (cat# C101) were purchased from Chematech and Vivotag™680XL-MAL (prod# NEV11220) was purchased from PerkinElmer. 1H NMR spectra were recorded using a Bruker Avance 300 MHz spectrometer at 300 MHz. Chemical shifts (δ) are reported in ppm and interaction constants (J) in Hz. The spectra were referenced to the residual solvent signals. Mass spectra (LCMS) of the synthetic products were obtained using Quattro Micro (Micromass, USA) spectrometer equipped with electro-spray ionization in positive mode (ESI+). The purity of the prepared compounds was >95% as determined by the combination of HPLC (H2O/acetonitrile, linear gradient) and 1H NMR. The DNA oligonucleotides (ODNs) were purchased from IDT Technologies as desalted ODNs. The H_2O used for the DNA experiments was purified on a MilliQ system. Size-exclusion chromatography (SEC) and reverse phase (RP) analysis of PEGylated Her-Fab were performed with Waters Acquity class H UPLC systems fitted with PDA detector according to the following conditions: SEC UPLC was run on a BEH200 SEC 1.7 µm, 4.6 x 150 mm column operated at 40 °C, flow 0.4 mL/min with an isocratic gradient of 5% isopropanol in PBS buffer, pH 7.4 for 12 minutes. RP UPLC was run on a BEH C4 1.7 µm, 2.1 x 50 mm column operated at 40 °C, flow 0.4 mL/min, line A: 0.05% trifluoroacetic acid (TFA) in MQ water, line B: 0.05% TFA in acetonitrile, linear gradient 25% to 75% B over 5 min. RP-HPLC for analysis and purification of oligonucleotides was performed on a Hewlett Packard Agilent 1100 or 1200 Series using Phenomenex Clarity 3u Oligo-RP with a 4.6 mm x 50 mm column, 3.6 µm, 100 Å. All concentrations of oligonucleotides and proteins were determined using a Thermo Fisher Scientific ND-1000 NanoDrop spectrophotometer and a Thermo Fisher Qbit 3™ Protein Assay. LC-MS of all the intermediate and final products during the development of the labelling protocol with the reagents were acquired with an Agilent 6230 TOF instrument connected to an Agilent 1290 Infinity UPLC fitted with an Aeris Widepore C4, 3.6 µm, 2.1 x 50 mm columns operated at 40 °C, flow 0.4 mL/min, line A: 0.02% TFA in MQ water, line B: 0.02% TFA in acetonitrile, 20% to 90% B linear gradient over 7 min. General SDS-PAGE gel-analyses were performed using the NuPage™ system from Thermo Scientific. Protein samples were prepared by addition of NuPage™ LDS sample buffer (4X) and incubated at 70 °C for 10 min. prior to analysis with pre-cast Novex™ 4-12% Bis-Tris gels. Reduction of samples was performed with NuPAGE® Sample Reducing Agent (10X) and, unless otherwise noted, a SeeBlue Plus 2 size marker was employed. Prior to analysis, the gels were stained for protein with SimplyBlueTM SafeStain (Life TechnologiesTM) and imaged with a Gel Doc™ EZ (Bio-Rad) or Amersham Typhoon 5 Biomolecular Imager in OD mode. Final image analysis was carried out in ImageLab.

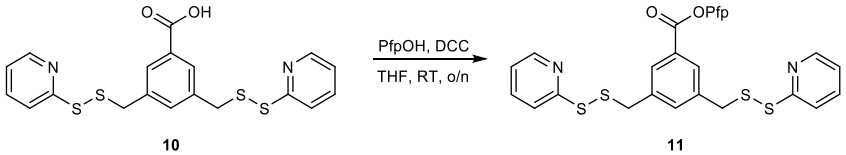
Organic Synthesis

Reagent **1: *N*-(2-((2-(4-Formylbenzamido)ethyl)disulfaneyl)ethyl)-3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzamide (1)**



1

Perfluorophenyl 3,5-bis((pyridin-2-yldisulfanyl)methyl)benzoate (11)



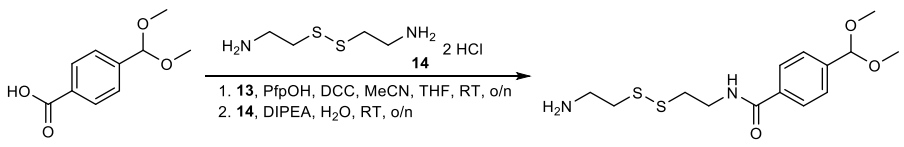
10 **11**

3,5-Bis((pyridin-2-yldisulfanyl)methyl)benzoic acid (**10**, 2.89 g, 6.68 mmol) was dissolved in tetrahydrofuran (40 mL) followed by addition of 2,3,4,5,6-pentafluorophenol (1.23 g, 6.68 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (1.38 g, 6.68 mmol). The resulting mixture was stirred overnight at room temperature before the precipitate was filtered off and the filtrate evaporated. The residue was dissolved in acetonitrile (40 mL) and residual *N*,*N*′-dicyclohexylurea was filtered off. The filtrate was evaporated to dryness affording perfluorophenyl 3,5-bis((pyridin-2-yldisulfanyl)methyl)benzoate (**11**) as a yellow oil.

Yield: 4.00 g (100%).

^{1}H NMR (300 MHz, DMSO-d_6, δ_H): 8.40-8.34 (m, 2 H); 7.92 (d, J=1.5 Hz, 2 H); 7.76-7.73 (m, 1 H); 7.70-7.62 (m, 2 H); 7.52-7.46 (m, 2 H); 7.19-7.12 (m, 2 H); 4.21 (s, 4 H).

***N*-(2-((2-aminoethyl)disulfanyl)ethyl)-4-(dimethoxymethyl)benzamide (15)**



13 **15**

4-(Dimethoxymethyl)benzoic acid (**13**, 3.00 g, 15.3 mmol) was dissolved in a acetonitrile/tetrahydrofuran mixture (6:1, 70 mL) followed by addition of 2,3,4,5,6-pentafluorophenol (2.82 g, 15.3 mmol) and *N*,*N*′-

dicyclohexylcarbodiimide (3.16 g, 15.3 mmol). The resulting mixture was stirred overnight at room temperature. The precipitate was then filtered off and the filtrate added to a solution of cystamine hydrochloride (**14**, 6.90 g, 30.6 mmol) and *N*,*N*-diisopropylethylamine (26.6 mL, 153 mmol) in water (50 mL). This mixture was stirred overnight at room temperature. Ethyl acetate (100 mL) was added and the phases were separated. The organic layer was washed with water (2 x 50 mL), dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was purified by flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol 95:5) to yield *N*-(2-((2-aminoethyl)disulfanyl)ethyl)-4-(dimethoxymethyl)benzamide (**15**) as a yellow oil.

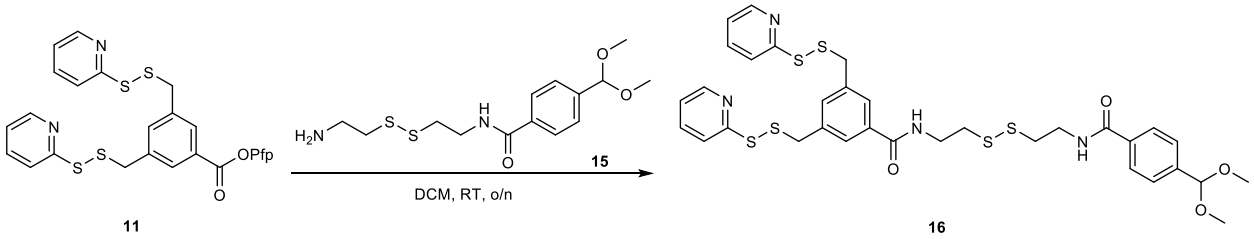
Yield: 3.32 g (66%).

R_F (SiO_2, dichloromethane/methanol 90:10): 0.55.

^{1}H NMR (300 MHz, $CDCl_3$, δ_H): 7.79 (d, J=8.4 Hz, 2 H); 7.53 (d, J=8.3 Hz, 2 H); 6.75-6.68 (m, 1 H); 5.43 (s, 1 H); 3.85-3.76 (m, 2 H); 3.03 (t, J=6.1 Hz, 2 H); 2.93 (t, J=6.1 Hz, 2 H); 2.79 (t, J=6.1 Hz, 2 H); 1.43 (bs, 2 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 5:95 to 100:0 + 0.1% FA): 2.18 min. LC-MS m/z: 331.1 $(M+H)^+$, calc. 330.11 (Exact Mass).

N-(2-((2-(4-(dimethoxymethyl)benzamido)ethyl)disulfaneyl)ethyl)-3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzamide (16)



11 16

Perfluorophenyl 3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzoate (**11**, 848 mg, 1.42 mmol) was dissolved in dichloromethane (20 mL) followed by addition of *N*-(2-((2-aminoethyl)disulfanyl)ethyl)-4-(dimethoxymethyl)benzamide (**15**, 468 mg, 1.42 mmol) dissolved in dichloromethane (20 mL). The resulting mixture was stirred overnight at room temperature. Then the solvent was evaporated and the residue was purified by flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol 98:2) to yield *N*-(2-((2-(4-(dimethoxymethyl)benzamido)ethyl)disulfaneyl)ethyl)-3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzamide (**16**) as a colorless tough oil.

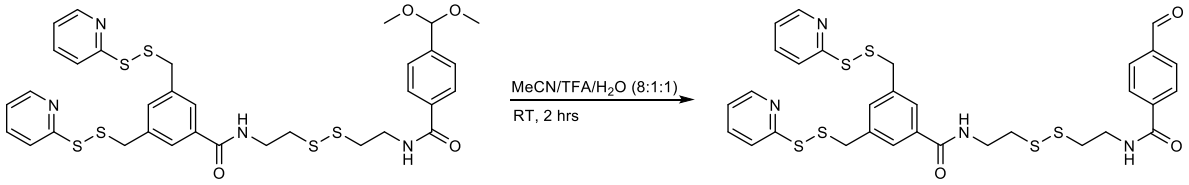
Yield: 961 mg (91%).

R_F (SiO_2, dichloromethane/methanol 95:5): 0.30.

^{1}H NMR (300 MHz, $CDCl_3$, δ_H): 8.43-8.38 (m, 2 H); 7.89-7.81 (m, 2 H); 7.61-7.43 (m, 8 H); 7.38-7.33 (m, 1 H); 7.14-6.99 (m, 4 H); 5.42 (s, 1 H); 3.94 (s, 4 H); 3.87-3.73 (m, 4 H); 3.32 (s, 6 H); 3.06-2.96 (m, 4 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 20:80 to 100:0 + 0.1% FA): 3.69 min. LC-MS m/z: 745.0 $(M)^+$, calc. 744.11 (Exact Mass).

N-(2-((2-(4-Formylbenzamido)ethyl)disulfaneyl)ethyl)-3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzamide, Reagent 1, 0 x AEEA



16 1

N-(2-((2-(4-(Dimethoxymethyl)benzamido)ethyl)disulfaneyl)ethyl)-3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzamide (**16**, 951 mg, 1.29 mmol) was dissolved in acetonitrile/trifluoroacetic acid/water mixture (8:1:1, 50 mL) and incubated for 2 hours. The solvent was evaporated and co-evaporated with acetonitrile three times. The residue was filtered through the short pad of silica (Silicagel 60, 0.040-0.063 mm; eluent: ethyl acetate) affording *N*-(2-((2-(4-formylbenzamido)ethyl)disulfaneyl)ethyl)-3,5-bis((pyridin-2-yldisulfaneyl)methyl)benzamide (**1**) as a pale yellow tough oil.

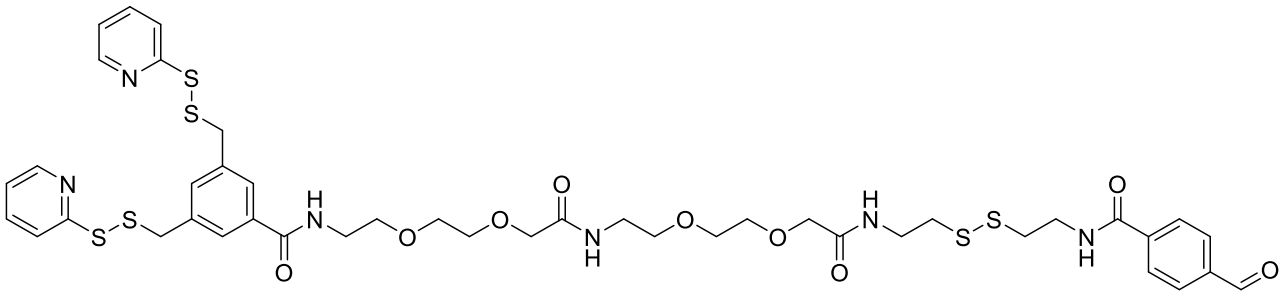
Yield: 902 mg (100%).

R_F (SiO_2, ethyl acetate): 0.50.

^{1}H NMR (300 MHz, AcOD-d_4, δ_H): 10.04 (s, 1 H); 8.53 (d, J=5.1 Hz, 2 H); 8.09-7.94 (m, 4 H); 7.90-7.80 (m, 2 H); 7.74-7.61 (m, 4 H); 7.44-7.31 (m, 3 H); 4.03 (s, 4 H); 3.88-3.71 (m, 4 H); 3.09-2.95 (m, 4 H).

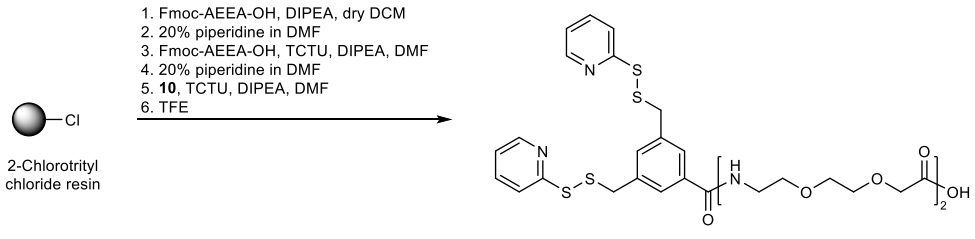
LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 35:65 to 100:0 + 0.1% FA): 3.00 min. LC-MS m/z: 699.0 $(M+H)^+$, calc. 698.06 (Exact Mass).

Reagent **2: *N*-(1-(4-Formylphenyl)-1,10,19-trioxo-12,15,21,24-tetraoxa-5,6-dithia-2,9,18-triazahexacosan-26-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (2)**



2

1-(3,5-bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10-dioxo-5,8,14,17-tetraoxa-2,11-diazanonadecan-19-oic acid (17)



17

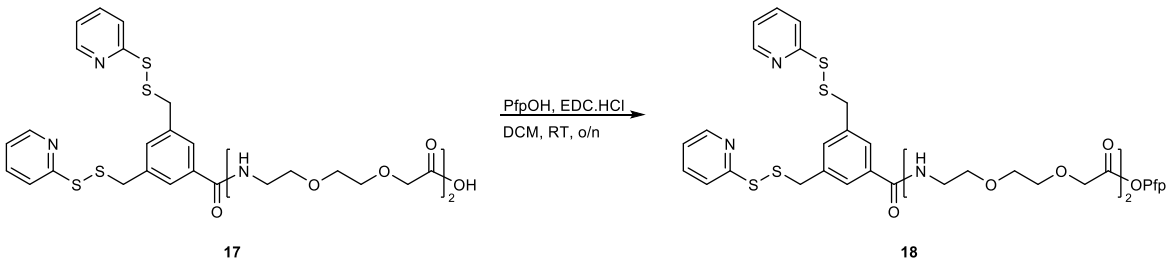
A 2-Chlorotrityl chloride resin 100-200 mesh 1.5 mmol/g (4.84 g, 7.30 mmol) was left to swell in dry dichloromethane (40 mL) for 20 minutes. A solution of {2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-acetic acid (Fmoc-AEEA-OH, 1.87 g, 4.80 mmol) and *N,N*-diisopropylethylamine (3.20 mL, 18.4 mmol) in dry dichloromethane (30 mL) was added to the resin and the mixture was incubated while shaking overnight. The resin was filtered and treated with a solution of *N,N*-diisopropylethylamine (1.69 mL, 9.70 mmol) in a methanol/dichloromethane mixture (1:4, 1 x 10 min, 1 x 40 mL) before it was washed with dichloromethane (2 x 40 mL) and *N,N*-dimethylformamide (2 x 40 mL). The Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (1 x 5 min, 1 x 20 min, 2 x 40 mL). The resin was washed with *N,N*-dimethylformamide (2 x 40 mL), 2-propanol (2 x 40 mL), dichloromethane (2 x 40 mL), and *N,N*-dimethylformamide (2 x 40 mL). A solution of {2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-acetic acid (Fmoc-AEEA-OH, 3.73 g, 9.70 mmol), 5-chloro-1-((dimethylamino)(dimethyliminio)methyl)-1*H*-benzo[*d*][1,2,3]triazole 3-oxide tetrafluoroborate (TCTU, 3.44 g, 9.70 mmol) and *N,N*-diisopropylethylamine (3.04 mL, 17.4 mmol) in *N,N*-dimethylformamide (30 mL) was added to the resin. The mixture was incubated while shaking for 2 hours before the resin was washed with *N,N*-dimethylformamide (2 x 40 mL), dichloromethane (2 x 40 mL), and *N,N*-dimethylformamide (2 x 40 mL). The Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (1 x 5 min, 1 x 20 min, 2 x 40 mL) prior to washing with *N,N*-dimethylformamide (2 x 40 mL), 2-propanol (2 x 40 mL), dichloromethane (2 x 40 mL), and *N,N*-dimethylformamide (2 x 40 mL). A solution of 3,5-bis((pyridin-2-yldisulfanyl)methyl)benzoic acid (**10**, 3.14 g, 7.30 mmol), 5-chloro-1-((dimethylamino)(dimethyliminio)methyl)-1*H*-benzo[*d*][1,2,3]triazole 3-oxide tetrafluoroborate (TCTU, 2.58 g, 7.30 mmol), and *N,N*-diisopropylethylamine (2.28 mL, 13.1 mmol) in *N,N*-dimethylformamide (40 mL) was added to the resin and incubated while shaking for 3 hours. The resin was washed with *N,N*-dimethylformamide (2 x 50 mL) and dichloromethane (10 x 50 mL). The product was cleaved overnight from the resin with 2,2,2-trifluoroethanol (80 mL). The resin was filtered and washed with dichloromethane (2 x 100 mL). Solvents were removed under reduced pressure and the residue was purified by flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol/acetic acid 89.5:10:0.5) to yield 1-(3,5-bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10-dioxo-5,8,14,17-tetraoxa-2,11-diazanonadecan-19-oic acid (**17**) as a pale yellow oil.

Yield: 2.37 g (68%).

^{1}H NMR (300 MHz, $CDCl_3$, δ_H): 8.43-8.35 (m, 2 H); 7.59-7.44 (m, 6 H); 7.40-7.30 (m, 2 H); 7.25-7.18 (m, 1 H); 7.08-6.99 (m, 2 H); 4.21 (s, 2 H); 4.01 (s, 2 H); 3.97 (s, 4 H); 3.81-3.57 (m, 14 H); 3.54-3.46 (m, 2 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 20:80 to 100:0 + 0.1% FA): 2.55 min. LC-MS m/z: 722.9 $(M+H)^+$, calc. 722.16 (Exact Mass).

Perfluorophenyl 1-(3,5-bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10-dioxo-5,8,14,17-tetraoxa-2,11-diazanonadecan-19-oate (18)



17 18

1-(3,5-Bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10-dioxo-5,8,14,17-tetraoxa-2,11-diazanonadecan-19-oic acid (**17**, 2.36 g, 3.26 mmol) was dissolved in dichloromethane (40 mL) followed by addition of 2,3,4,5,6-pentafluorophenol (0.72 g, 3.91 mmol) and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl, 0.81 g, 4.24 mmol). The resulting mixture was stirred overnight at room temperature before the

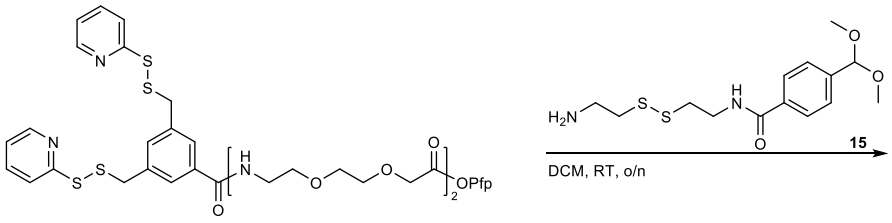
solvent was evaporated. The residue was dissolved in ethyl acetate (150 mL) and washed with water (3 x 100 mL) and brine (1 x 100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to give perfluorophenyl 1-(3,5-bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10-dioxo-5,8,14,17-tetraoxa-2,11-diazanonadecan-19-oate (**18**) as a pale orange oil.

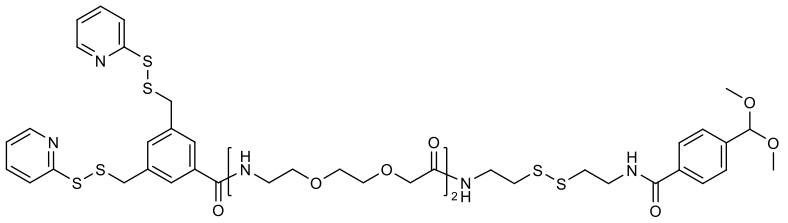
Yield: 2.80 g (97%).

^{1}H NMR (300 MHz, $CDCl_3$, δ_H): 8.45-8.39 (m, 2 H); 7.60-7.46 (m, 6 H); 7.38-7.34 (m, 1 H); 7.24-7.15 (m, 1 H); 7.09-7.01 (m, 2 H); 6.89-6.80 (m, 1 H); 4.51 (s, 2 H); 4.03 (s, 2 H); 3.98 (s, 4 H); 3.82-3.76 (m, 2 H); 3.75-3.63 (m, 10 H); 3.61-3.55 (m, 2 H); 3.51-3.43 (m, 2 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 35:65 to 100:0 + 0.1% FA): 3.17 min. LC-MS m/z: 888.9 $(M+H)^+$, calc. 888.14 (Exact Mass).

***N*-(1-(4-(Dimethoxymethyl)phenyl)-1,10,19-trioxo-12,15,21,24-tetraoxa-5,6-dithia-2,9,18-triazahexacosan-26-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (19)**





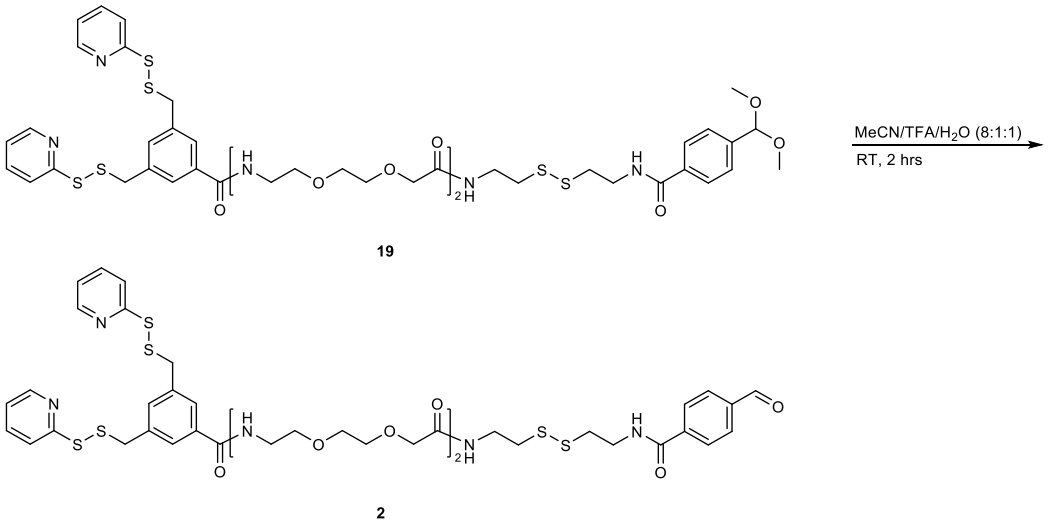
Perfluorophenyl 1-(3,5-bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10-dioxo-5,8,14,17-tetraoxa-2,11-diazanonadecan-19-oate (**18**, 2.80 g, 3.15 mmol) was dissolved in dichloromethane (40 mL) followed by addition of *N*-(2-((2-aminoethyl)disulfanyl)ethyl)-4-(dimethoxymethyl)benzamide (**15**, 1.15 g, 3.47 mmol). The resulting mixture was stirred overnight at room temperature before the solvent was evaporated. The residue was purified by flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol 97:3 to 95:5) to yield *N*-(1-(4-(dimethoxymethyl)phenyl)-1,10,19-trioxo-12,15,21,24-tetraoxa-5,6-dithia-2,9,18-triazahexacosan-26-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (**19**) as a pale yellow tough oil.

Yield: 0.68 g (21%). R_F (SiO_2, dichloromethane/methanol 90:10): 0.55.

^{1}H NMR (300 MHz, $CDCl_3$, δ_H): 8.45-8.38 (m, 2 H); 7.85 (d, J=8.3 Hz, 2 H); 7.61-7.47 (m, 6 H); 7.38-7.33 (m, 1 H); 7.32-7.23 (m, 2 H); 7.21-7.13 (m, 1 H); 7.09-7.03 (m, 2 H); 7.02-6.95 (m, 1 H); 5.42 (s, 1 H); 4.03-3.93 (m, 8 H); 3.80-3.54 (m, 18 H); 3.51-3.44 (m, 2 H); 3.32 (s, 6 H); 2.97 (t, J=6.1 Hz, 2 H); 2.85 (t, J=6.5 Hz, 2 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 35:65 to 100:0 + 0.1% FA): 2.46 min. LC-MS m/z: 1035.3 $(M+H)^+$, calc. 1034.25 (Exact Mass).

***N*-(1-(4-Formylphenyl)-1,10,19-trioxo-12,15,21,24-tetraoxa-5,6-dithia-2,9,18-triazahexacosan-26-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide, Reagent 2, 2 x AEEA.**



19

2

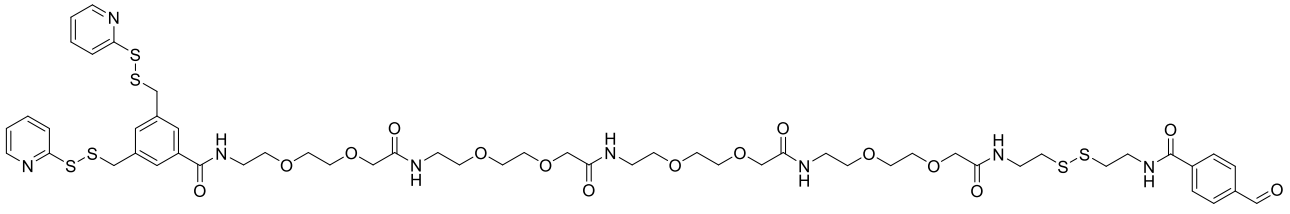
N-(1-(4-(Dimethoxymethyl)phenyl)-1,10,19-trioxo-12,15,21,24-tetraoxa-5,6-dithia-2,9,18-triazahexacosan-26-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (**19**, 0.67 g, 0.65 mmol) was dissolved in a acetonitrile/trifluoroacetic acid/water mixture (8:1:1, 40 mL) and incubated for 2 hours. Then the solvents were evaporated and co-evaporated with acetonitrile three times. The residue was dissolved in dichloromethane (15 mL) and molecular sieves were added. This mixture was incubated for 2 hours before the sieves were filtered off. The filtrate was evaporated to dryness to afford *N*-(1-(4-formylphenyl)-1,10,19-trioxo-12,15,21,24-tetraoxa-5,6-dithia-2,9,18-triazahexacosan-26-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (**2**) as pale a yellow tough oil.

Yield: 568 mg (89%).

^{1}H NMR (300 MHz, $CDCl_3$/AcOD-d_4, δ_H): 10.02 (s, 1 H); 8.52-8.43 (m, 2 H); 8.01-7.86 (m, 4 H); 7.76-7.66 (m, 2 H); 7.64-7.53 (m, 4 H); 7.31 (s, 1 H); 7.24-7.16 (m, 2 H); 4.07-3.89 (m, 8 H); 3.79-3.51 (m, 18 H); 3.48-3.40 (m, 2 H); 2.95 (t, J=5.5 Hz, 2 H); 2.83 (t, J=6.1 Hz, 2 H).

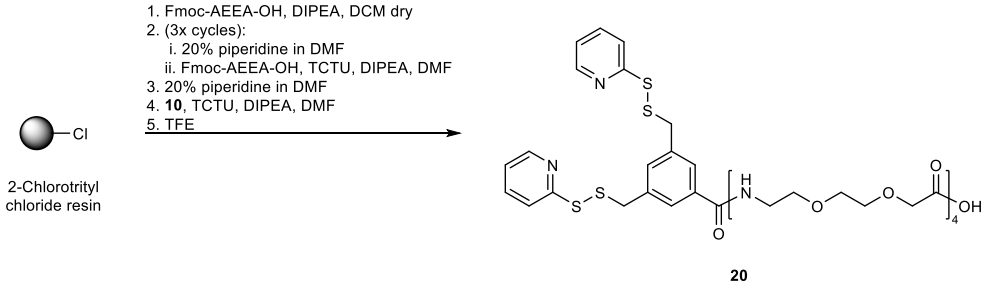
LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 35:65 to 100:0 + 0.1% FA): 2.30 min. LC-MS m/z: 989.2 $(M+H)^+$, calc. 988.21 (Exact Mass).

Reagent **3: *N*-(1-(4-Formylphenyl)-1,10,19,28,37-pentaoxo-12,15,21,24,30,33,39,42-octaoxa-5,6-dithia-2,9,18,27,36-pentaazatetratetracontan-44-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (3)**



3

1-(3,5-Bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10,19,28-tetraoxo-5,8,14,17,23,26,32,35-octaoxa-2,11,20,29-tetraazaheptatriacontan-37-oic acid (20)



20

A 2-Chlorotrityl chloride resin 100-200 mesh 1.5 mmol/g (12.8 g, 19.2 mmol) was left to swell in dry dichloromethane (90 mL) for 20 minutes. A solution of {2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-acetic acid (Fmoc-AEEA-OH, 2.09 g, 5.40 mmol) and *N,N*-diisopropylethylamine (3.59 mL, 20.6 mmol) in dry dichloromethane (80 mL) was added to the resin and incubated while shaking for 3 days. The resin was filtered and treated with a solution of *N,N*-diisopropylethylamine (1.89 mL, 10.9 mmol) in a methanol/dichloromethane mixture (1:4, 1 x 15 min, 1 x 50 mL). The resin was washed with *N,N*-dimethylformamide (2 x 80 mL), dichloromethane (2 x 80 mL), and *N,N*-dimethylformamide (2 x 80 mL) before the Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (1 x 5 min, 1 x 20 min, 2 x 50 mL). The resin was then washed with *N,N*-dimethylformamide (2 x 80 mL), 2-propanol (2 x 80 mL), dichloromethane (2 x 80 mL), and *N,N*-dimethylformamide (2 x 80 mL). A solution of {2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-acetic acid (Fmoc-AEEA-OH, 4.18 g, 10.9 mmol), 5-chloro-1-((dimethylamino)(dimethyliminio)methyl)-1*H*-benzo[d][1,2,3]triazole 3-oxide tetrafluoroborate (TCTU, 3.86 g, 10.9 mmol), and *N,N*-diisopropylethylamine (3.40 mL, 19.5 mmol) in *N,N*-dimethylformamide (50 mL) was added to the resin and incubated for 2.5 hours while shaking. The resin was washed with *N,N*-dimethylformamide (2 x 80 mL), dichloromethane (2 x 80 mL), and *N,N*-dimethylformamide (2 x 80 mL). The Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (1 x 5 min, 1 x 20 min, 2 x 50 mL) before washing with *N,N*-dimethylformamide (2 x 80 mL), 2-propanol (2 x 80 mL), dichloromethane (2 x 80 mL) and *N,N*-dimethylformamide (2 x 80 mL). A solution of {2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-acetic acid (Fmoc-AEEA-OH, 4.18 g, 10.9 mmol), 5-chloro-1-((dimethylamino)(dimethyliminio)methyl)-1*H*-benzo[d][1,2,3]triazole 3-oxide tetrafluoroborate (TCTU, 3.86 g, 10.9 mmol), and *N,N*-diisopropylethylamine (3.40 mL, 19.5 mmol) in *N,N*-dimethylformamide (50 mL) was added to the resin and the mixture was shaken for 2.5 hours. The resin was washed with *N,N*-dimethylformamide (2 x 80 mL), dichloromethane (2 x 80 mL), and *N,N*-dimethylformamide (2 x 80 mL). The Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (1 x 5 min, 1 x 20 min, 2 x 50 mL). The resin was washed with *N,N*-dimethylformamide (2 x 80 mL), 2-propanol (2 x 80 mL), dichloromethane (2 x 80 mL), and *N,N*-dimethylformamide (2 x 80 mL). A solution of {2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-acetic acid (Fmoc-AEEA-OH, 4.18 g, 10.9 mmol), 5-chloro-1-((dimethylamino)(dimethyliminio)methyl)-1*H*-benzo[d][1,2,3]triazole 3-oxide tetrafluoroborate (TCTU, 3.86 g, 10.9 mmol), and *N,N*-diisopropylethylamine (3.40 mL, 19.5 mmol) in *N,N*-dimethylformamide (50 mL) was added to the resin and the mixture was shaken for 2 hours. The resin was washed with *N,N*-dimethylformamide (2 x 80 mL), dichloromethane (2 x 80 mL), and *N,N*-dimethylformamide (2 x 80 mL). The Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (1 x 5 min, 1 x 20

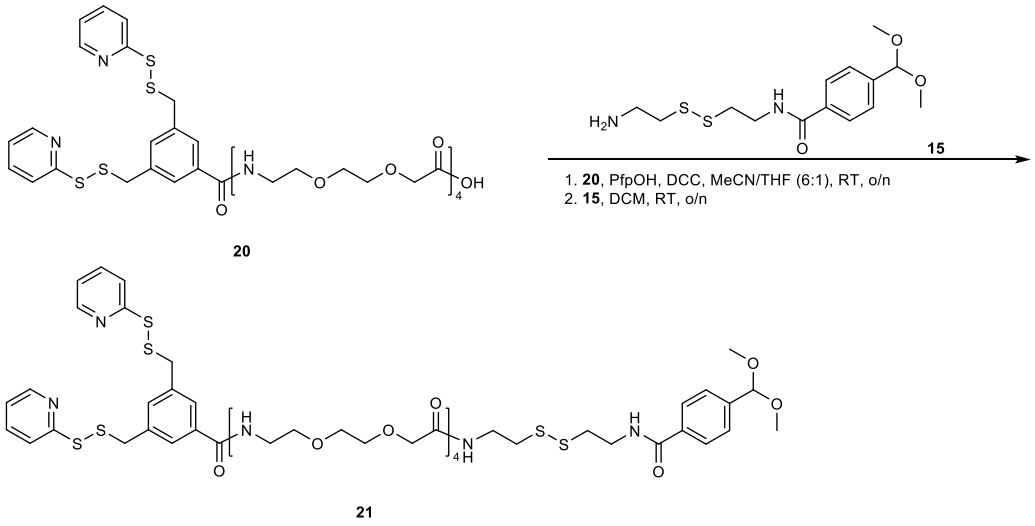
min, 2 x 50 mL). The resin was washed with *N,N*-dimethylformamide (2 x 80 mL), 2-propanol (2 x 80 mL), dichloromethane (2 x 80 mL) and *N,N*-dimethylformamide (2 x 80 mL). A solution of 3,5-bis((pyridin-2-yldisulfanyl)methyl)benzoic acid (**10**, 3.55 g, 8.21 mmol), 5-chloro-1-((dimethylamino)(dimethyliminio)methyl)-1*H*-benzo[d][1,2,3]triazole 3-oxide tetrafluoroborate (TCTU, 2.92 g, 8.21 mmol), and *N,N*-diisopropylethylamine (2.56 mL, 14.7 mmol) in *N,N*-dimethylformamide (60 mL) was incubated with the resin while shaking for 3 hours. The resin was washed with *N,N*-dimethylformamide (2 x 100 mL) and dichloromethane (10 x 100 mL) before the product was cleaved overnight from the resin by the treatment with 2,2,2-trifluoroethanol (80 mL). The resin was filtered and washed with dichloromethane (2 x 100 mL), solvents were removed under reduced pressure, and the residue was purified by flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol 80:20) to yield 1-(3,5-bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10,19,28-tetraoxo-5,8,14,17,23,26,32,35-octaoxa-2,11,20,29-tetraazaheptatriacontan-37-oic acid (**20**) as a yellow tough oil.

Yield: 4.03 g (73%).

^{1}H NMR (300 MHz, DMSO-d_6, δ_H): 8.49-8.39 (m, 3 H); 7.75-7.55 (m, 9 H); 7.44 (t, J=1.6 Hz, 1 H); 7.22-7.15 (m, 2 H); 4.11 (s, 4 H); 3.99 (s, 2 H); 3.88 (s, 2 H); 3.87 (s, 4 H); 3.59-3.48 (m, 15 H); 3.47-3.36 (m, 9 H); 3.33-3.22 (m, 8 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C8, 4.6 mm x 50 mm, acetonitrile/water 20:80 to 100:0 + 0.1% FA): 2.83 min. LC-MS m/z: 1013.2 $(M+H)^+$, calc. 1012.31 (Exact Mass).

_N_-(1-(4-(Dimethoxymethyl)phenyl)-1,10,19,28,37-pentaoxo-12,15,21,24,30,33,39,42-octaoxa-5,6-dithia-2,9,18,27,36-pentaazatetratetracontan-44-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (21)



1-(3,5-Bis((pyridin-2-yldisulfanyl)methyl)phenyl)-1,10,19,28-tetraoxo-5,8,14,17,23,26,32,35-octaoxa-2,11,20,29-tetraazaheptatriacontan-37-oic acid (**20**, 0.99 g, 0.98 mmol) was dissolved in tetrahydrofuran (20 mL) followed by addition of 2,3,4,5,6-pentafluorophenol (0.18 g, 0.98 mmol) and *N,N*-dicyclohexylcarbodiimide (0.20 g, 0.98 mmol) and the resulting mixture was stirred overnight at room temperature. The precipitate was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in dichloromethane (10 mL) and a solution of *N*-(2-((2-aminoethyl)disulfanyl)ethyl)-4-(dimethoxymethyl)benzamide (**15**, 0.36 g, 1.10 mmol) in dichloromethane (10 mL) was added. The resulting mixture was stirred overnight at room temperature before the solvent was evaporated. The residue was purified by 2 rounds of flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol 95:5 to 91:9 then dichloromethane/methanol 98:2 to 90:10) to yield *N*-(1-(4-

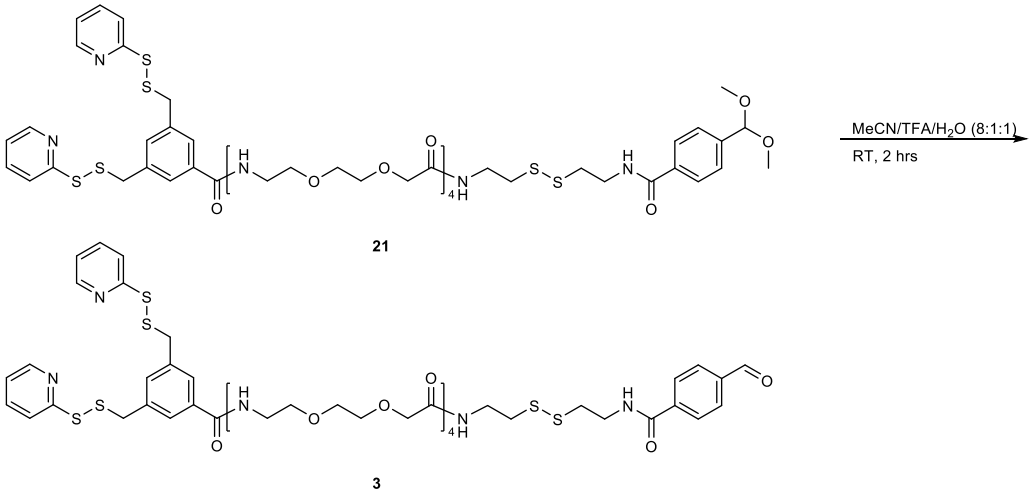
(dimethoxymethyl)phenyl)-1,10,19,28,37-pentaoxo-12,15,21,24,30,33,39,42-octaoxa-5,6-dithia-2,9,18,27,36-pentaazatetratetracontan-44-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (**21**) as a colorless oil.

Yield: 561 mg (43%).

R_F (SiO_2, dichloromethane/methanol 90:10): 0.45.

^{1}H NMR (300 MHz, $CDCl_3$, δ_H): 8.52-8.25 (m, 3 H); 7.95-7.80 (m, 3 H); 7.69-7.47 (m, 9 H); 7.39-7.32 (m, 2 H); 7.16-7.02 (m, 4 H); 5.43 (s, 1 H); 4.06-3.95 (m, 12 H); 3.74-3.44 (m, 36 H); 3.33 (s, 6 H); 3.04-2.97 (m, 2 H); 2.87 (t, J=6.8 Hz, 2 H).

***N*-(1-(4-Formylphenyl)-1,10,19,28,37-pentaoxo-12,15,21,24,30,33,39,42-octaoxa-5,6-dithia-2,9,18,27,36-pentaazatetratetracontan-44-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide, Reagent 3, 4 x AEEA**



N-(1-(4-(Dimethoxymethyl)phenyl)-1,10,19,28,37-pentaoxo-12,15,21,24,30,33,39,42-octaoxa-5,6-dithia-2,9,18,27,36-pentaazatetratetracontan-44-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (**21**, 0.56 g, 0.42 mmol) was dissolved in an acetonitrile/trifluoroacetic acid/water mixture (8:1:1, 40 mL) and incubated for 2 hours. The solvents were then evaporated and co-evaporated with acetonitrile three times before the residue was purified by 2 rounds of flash column chromatography (Silicagel 60, 0.040-0.063 mm; eluent: dichloromethane/methanol 98:2 to 90:10). Impure fractions were purified by preparative thin layer chromatography (eluent: dichloromethane/methanol 90:10). All the pure materials were combined together and dried *in vacuo* to yield *N*-(1-(4-formylphenyl)-1,10,19,28,37-pentaoxo-12,15,21,24,30,33,39,42-octaoxa-5,6-dithia-2,9,18,27,36-pentaazatetratetracontan-44-yl)-3,5-bis((pyridin-2-yldisulfanyl)methyl)benzamide (**3**) as a pale yellow oil.

Yield: 292 mg (54%).

R_F (SiO_2, dichloromethane/methanol 90:10): 0.40.

^{1}H NMR (300 MHz, MeOD-d_4, δ_H): 10.06 (s, 1 H); 8.34-8.29 (m, 2 H); 8.01-7.96 (m, 3 H); 7.86-7.75 (m, 1 H); 7.69-7.59 (m, 4 H); 7.58-7.52 (m, 2 H); 7.41 (s, 1 H); 7.16-7.08 (m, 2 H); 4.03-3.96 (m, 12 H); 3.72-3.62 (m, 20 H); 3.60-3.53 (m, 10 H); 3.47-3.40 (m, 6 H); 3.01-2.94 (m, 2 H); 2.92-2.85 (m, 2 H).

LC-MS purity: 100% (ELSD). LC-MS Rt (Kinetex C18, 4.6 mm x 50 mm, acetonitrile/water 20:80 to 100:0 + 0.1% FA): 3.23 min. LC-MS m/z: 640.7 $(M/2+H)^+$, calc. 1278,36 (Exact Mass).

Protocols

Preparation of Fab fragments

All mAbs were purchased as the pharmaceutical compound at a registered vendor in amounts that would not impact the supply for patients. Papain on beads was purchased through Thermo Fisher Scientific. NAb™ Spin Columns, 1mL Protein A Plus columns were purchased through Thermo Fisher Scientific.

Buffers for Papain digestion:

Sample buffer: Phosphate 20 mM, pH=7, 10 mM EDTA,
Digestion buffer: Sample buffer with 20 mM L-Cysteine (cysteine added just before use)
Removal Buffer: 10 mM TRIS*HCl, pH = 7.5

Buffers for Protein A binding:

Binding buffer: 0.1M phosphate, 0.15M sodium chloride; pH 7.2
Elution buffer: 0.1M glycine, pH 2-3
Storage Buffer:0.02% sodium azide in PBS
Neutralization Buffer: 1M phosphate pH 8, with 150 mM NaCl and 10 mM EDTA

Sample preparation:

All mAbs were transferred to sample buffer. mAbs supplied as the solid compound were dissolved directly in buffer, while mAbs in solution were BE with 10 mL Zeba spin desalting columns (40 kDa MWCO) to sample buffer.

General protocol for papain digestion of mAb

The protocol is based on the Thermo Fisher product protocol with the following changes: Papain on beads (1 mL) was added to a 15 mL tube along with 10 mL digestion buffer and centrifuged (1 min, 1000 RCF). The supernatant was removed and then washed twice with digestion buffer (10 mL, 1 min, 1000 RCF). Samples were then mixed 1:1 with digestion buffer. To 1 mL of Papain was added 1 mL of mAb and 2 mL digestion buffer. The samples were incubated ON at 37 °C and digestion confirmed by SDS-PAGE.* The mixture was filtered through a syringe filter. Lastly, the beads were then washed with TRIS-HCl removal buffer (2 x 1.5 mL, total 3 mL) and the filtrate (6 mL) was collected.

*Digestion of IgG2 based Vectibix was unsuccessful.

General protocol for Protein A binding

NAb Protein A columns were emptied and equilibrated (1000 rcf, 1 min) with binding buffer (2 x 2mL). The sample was diluted to 10 mL with Binding Buffer (4 mL) and added to 5x 2 mL NAb spin columns containing 1 mL Protein A on solid support. The samples were flipped end over end till the beads were dispersed and incubated for 10 minutes while rolling. The columns were eluted by centrifugation (1000 rcf, 1 min) and washed with binding buffer (2 mL). All fractions were then analysed by SDS-PAGE and product-containing fractions were pooled and purified by Äkta (isocratic, 5 mL loop, 60/200 superdex column, 2.5 mL/min, 320 min, buffer = 0.15 M PBS, pH=7.4). The product content of fractions was verified by SDS-PAGE before they were pooled. The sample purity was confirmed by LCMS. The Fab generally eluted around 200 min.

Protein modifications

Reaction Buffers:

Buffer A 10X Stock: 10X PBS buffer made with Gibco™ PBS tablets containing 20 mM EDTA, adjusted to pH=6.8 with HCl
Buffer A: 1X PBS, pH=6.8 buffer made with Gibco™ PBS tablets containing 2 mM EDTA
Buffer B: 0.1 M HEPES, pH = 7.5

General protocol for Fab reduction with DTT

DTT (20 µL, 0.5 M) was added to Fab (to 980 µL / 68,6 nmol) to a final concentration of protein ~4 mg/mL / 70 µM and DTT 10 mM. The reaction was incubated for 1 hour prior to buffer exchange (BE) with 2 mL Zeba™ spin desalting columns (7000 MWCO), which were equilibrated with Buffer A thrice, according to the supplier's instructions, to remove residual DTT.

Modification of Fab with pseudo-cysteine through reagent 1, 2 or 3

To the reduced Fab (62 µM, 331 µL) was added DMSO (20.75 µL) and then reagent for a final DMSO content of 20%* and reagent **1**, **2** or **3** in 3 eq. excess relative to Fab. The sample was then incubated for 30 minutes whereupon it was diluted to 1.8 mL with Buffer A and BE with 5 mL Zeba spin desalting columns (7 kDa MWCO) pre-equilibrated as per the supplier's instructions with Buffer A. After BE the sample was diluted with Buffer A (7 mL) and reductive amination conditions were established by addition of $NaBH_3CN$ (0.400 M, 1 mL) at a final concentration of 50 mM. The sample was incubated overnight (22 hours) at 37 °C. The reaction pH was checked to be 6.88. To release the guiding moiety and reveal the thiol handle, DTT (80 µL, 0.5 M) was added and incubated 1 hour. The samples were concentrated to 4 mL with Amicon Ultra centrifugal filters (MWCO 10k) by centrifugation (5 min, 4000 RCF). This was followed by BE with a 10 mL Zeba™ spin desalting column (7 kDa MWCO) to remove any remaining DTT. To each sample was added dehydroascorbic acid (dhAA, 10 mM, 64 µL, 40 eq.) for a final concentration of 160 µM dhAA to reoxidise the Fab to the final conjugate.

*for reagent **2** 10% DMSO is sufficient, and for reagent **3** 5% DMSO is sufficient.

Modification of hGH with pseudo-cysteine handle

To hGH in buffer A (316 µM, 1650 µL) was added TCEP (10 mM, 2.5 eq, 130 µL) for a final concentration of hGH of 292 µM and TCEP 730 µM. The sample was incubated for 45 minutes on the benchtop. To the reduced hGH (40 µL, 12 nmol) was added buffer A (670 µL) and reagent **2** or **3** (10 mM, 90 µL, 7 eq) for a final protein concentration of 16 µM, reagent concentration of 112.5 µM and DMSO content of 11%. The sample was then incubated for 30 minutes where after it was buffer exchanged with 5 mL Zeba spin desalting columns (7 kDa MWCO) that were equilibrated previously as per the supplier's instructions with Buffer A. After BE reductive amination conditions were established by addition of $NaBH_3CN$ (0.5 M, 80 µL) at a final concentration of 45 mM. The sample was incubated overnight (22 hours) at 37 °C. To release the guiding moiety and reveal the thiol handle DTT (80 µL, 0.5 M) was added and incubated 30 minutes. The samples were BE twice with Zeba spin desalting columns (7 kDa MWCO) that were equilibrated previously as per the supplier's instructions with Buffer A. The sample was treated dehydroascorbic acid (dhAA, 10 mM, 11.5 µL, 10 eq.) for a final concentration of 120 µM dhAA to furnish the reoxidised hGH conjugate.

Conjugation to pseudo-cysteine handle with NEM

To the pseudo-cysteine mutant of a Fab or hGH (typically at 4 µM) was added 20 eq. of NEM from a 100 mM stock in Buffer A. This was incubated for 20 minutes and the protein was washed (Buffer B) and filtered by Amicon Ultra Centrifugation filters (MWCO 10 kDa).

Conjugation to pseudo-cysteine handle with DNA and PEG

To the Fab or hGH with freshly installed thiol handle (typically at 4 µM) was added 1.2 eq. of DNA-maleimide or 2 eq. PEG-maleimide from a stock solution (100 µM), in Buffer A and the reaction was incubated for 60 minutes. Conjugates were purified on an Äkta FPLC (isocratic, 5 mL loop, 60/200 superdex column, 2.5 mL/min, 320 min, buffer = 0.15 M PBS, pH=7.4).

Preparation of ODN-maleimide

To ODN1 (100 nmol, 100 µL) was added Sulfo-SMCC in DMSO (100 mM, 15 µL) and 0.1M PBS pH 7.4 (35 µL) and the reaction was followed by RP-HPLC. After two additional hours, 0.5 mg SMCC was added. After 1 hour the DNA was precipitated with NaOAc (3 M, pH=5.2, 15 µL) and EtOH (495 µL) followed by centrifugation at 21000 RCF for 60 min. The precipitate was isolated and redissolved in MQ, hereafter it was purified by RP-HPLC on an Oligo Clarity column (A: 0.1 M triethylammonium acetate in MQ, B: acetonitrile, gradient: 5% to 30% B over 15 min) and the product with Rt =8.8 min was isolated and used without further purification.

Preparation for MS/MS

To remove phosphate buffer (that inhibits trypsin) from the proteins and concentrate them after conjugation with NEM, the samples were washed with buffer B (3 x 4 mL) in 4 mL Amicon Ultra centrifugal filters (10 kDa MWCO) and then concentrated to >2 mg/mL.

LCMS data

Her-Fab experiments

Native Fab

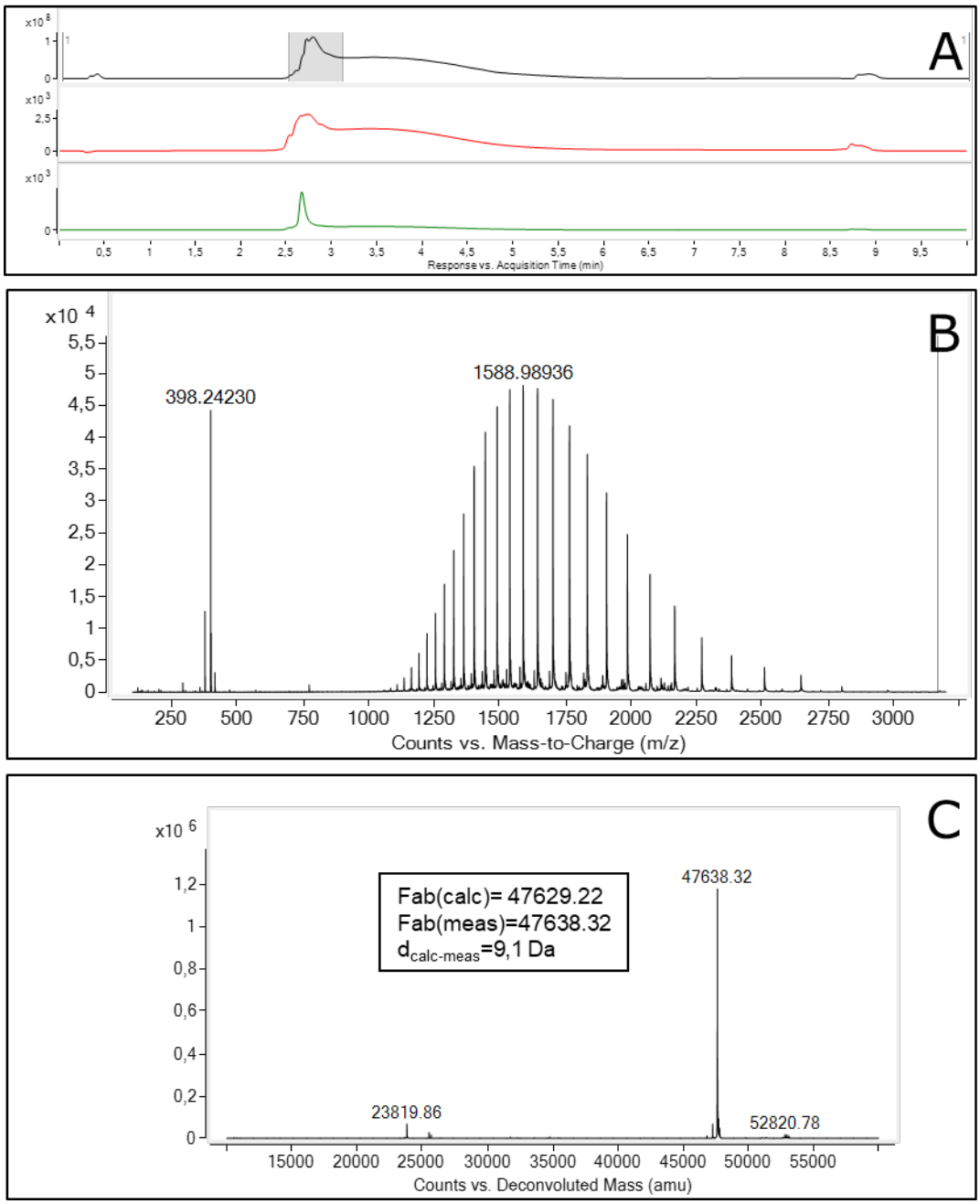


Figure S1. *The ÄKTA-purified Her-Fab following papain digestion of Herceptin® analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B.*

Reduced Fab

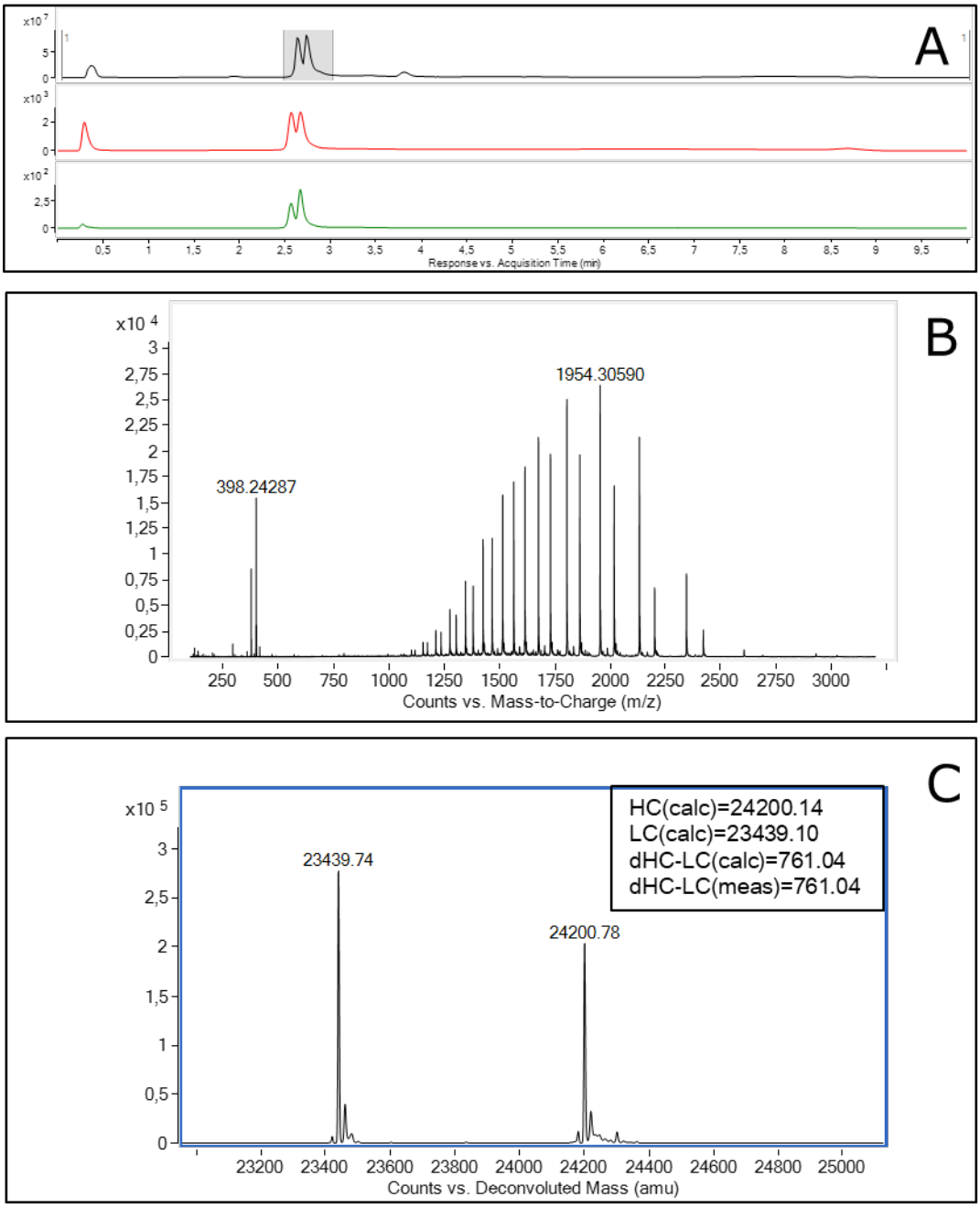


Figure S2. *The reduction of Her-Fab with DTT analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses of light chain and heavy chain indicated in the inset.*

Her-Fab reduction followed by NEM

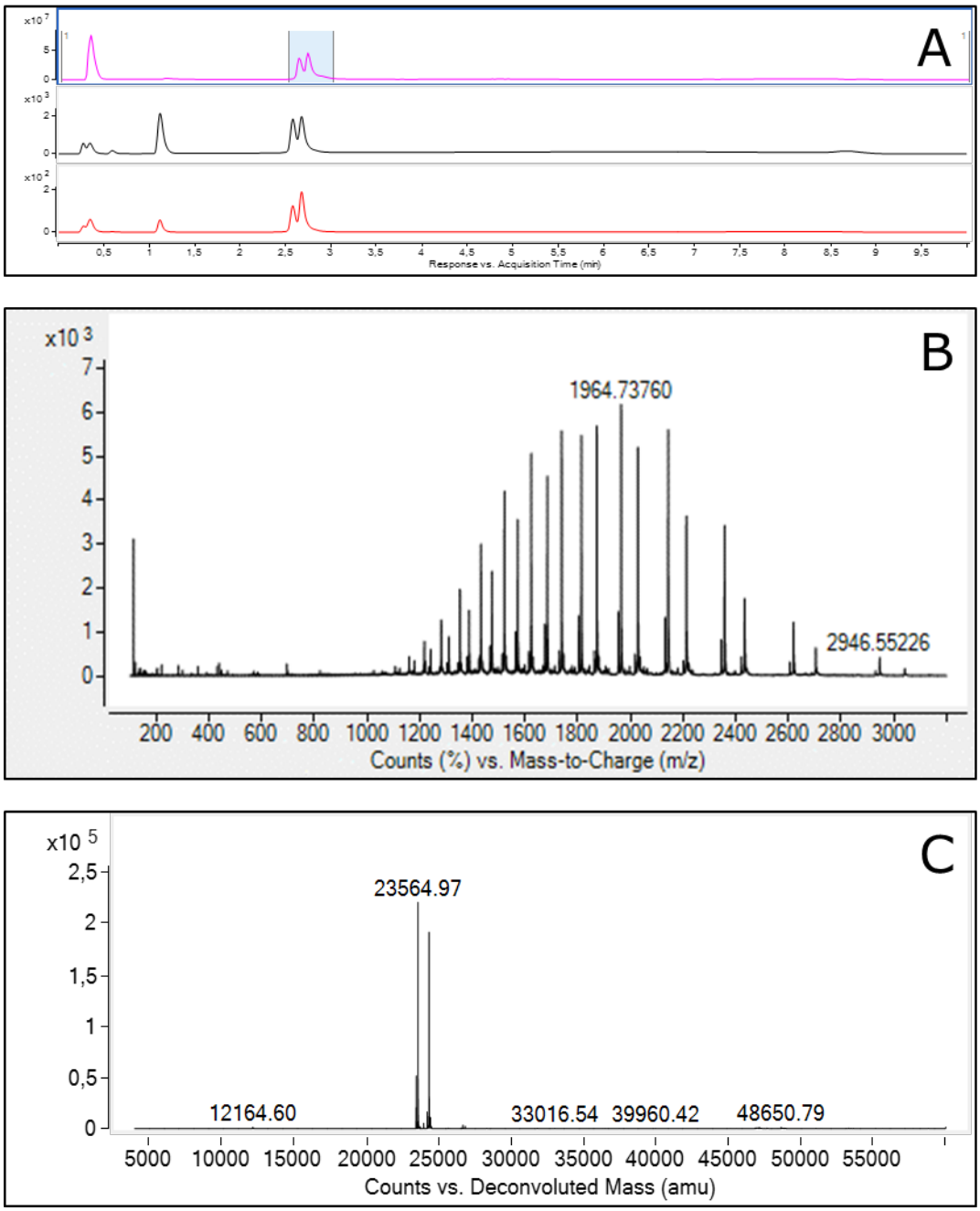


Figure S3. *The reduction of Her-Fab and subsequent addition of NEM analysed by LCMS. 1 molecule of NEM is added to both the heavy chain and light chain of the Fab corresponding to one opened disulphide. A) TIC (purple), DAD 214 nm (black), and DAD 280 nm (red). B) m/z of the grey scan range highlighted in A). C) Deconvolution of B showing the addition of +125 to each heavy and light chain.*

Her-Fab rebridged with reagent **1**

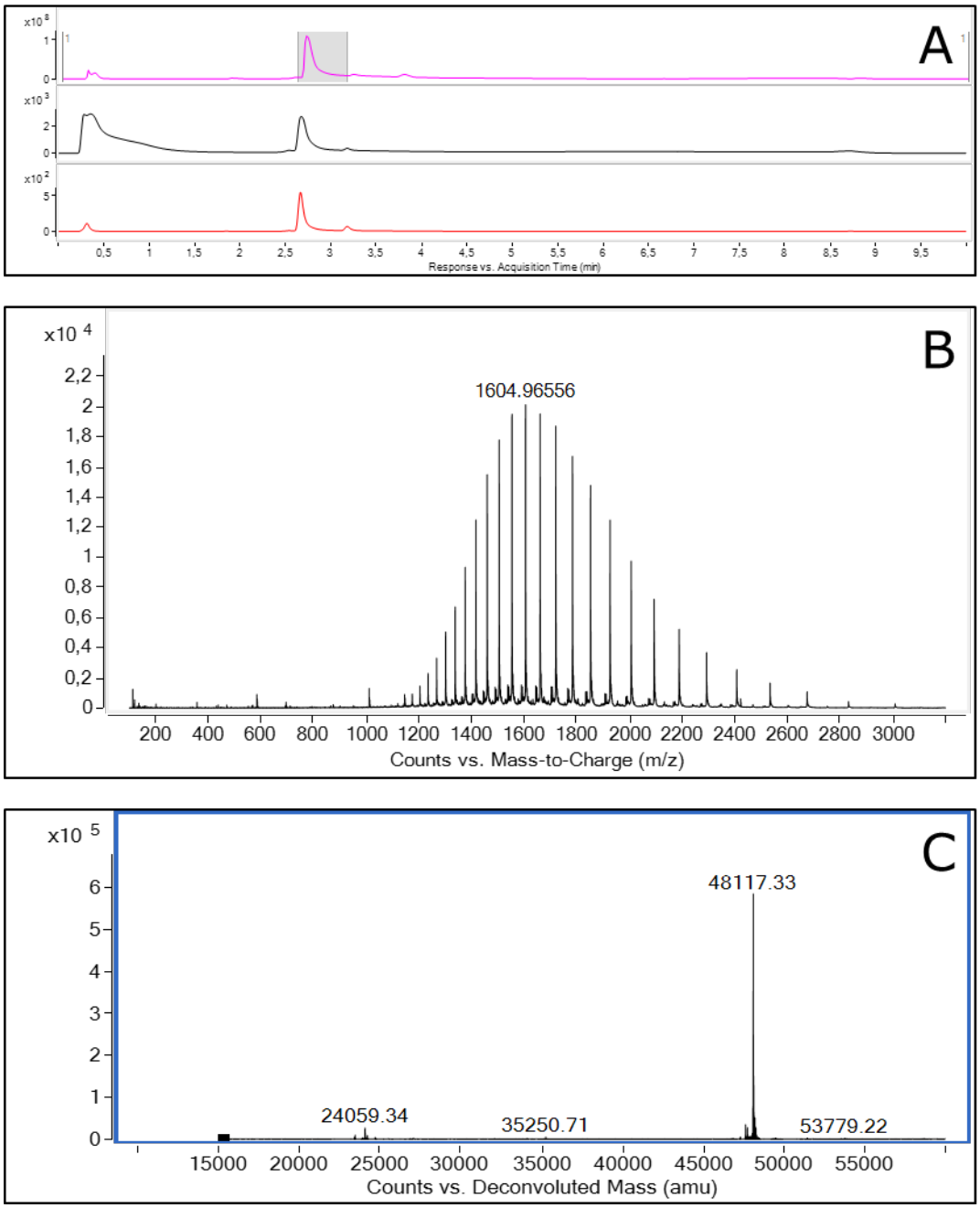


Figure S4. *The reaction between reduced Her-Fab and reagent **1** analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 48117.33 Da corresponds to Her-Fab rebridged with a single molecule of reagent **1** (Expected mass = 48117.38 Da).*

Her-Fab rebridged with reagent **2**

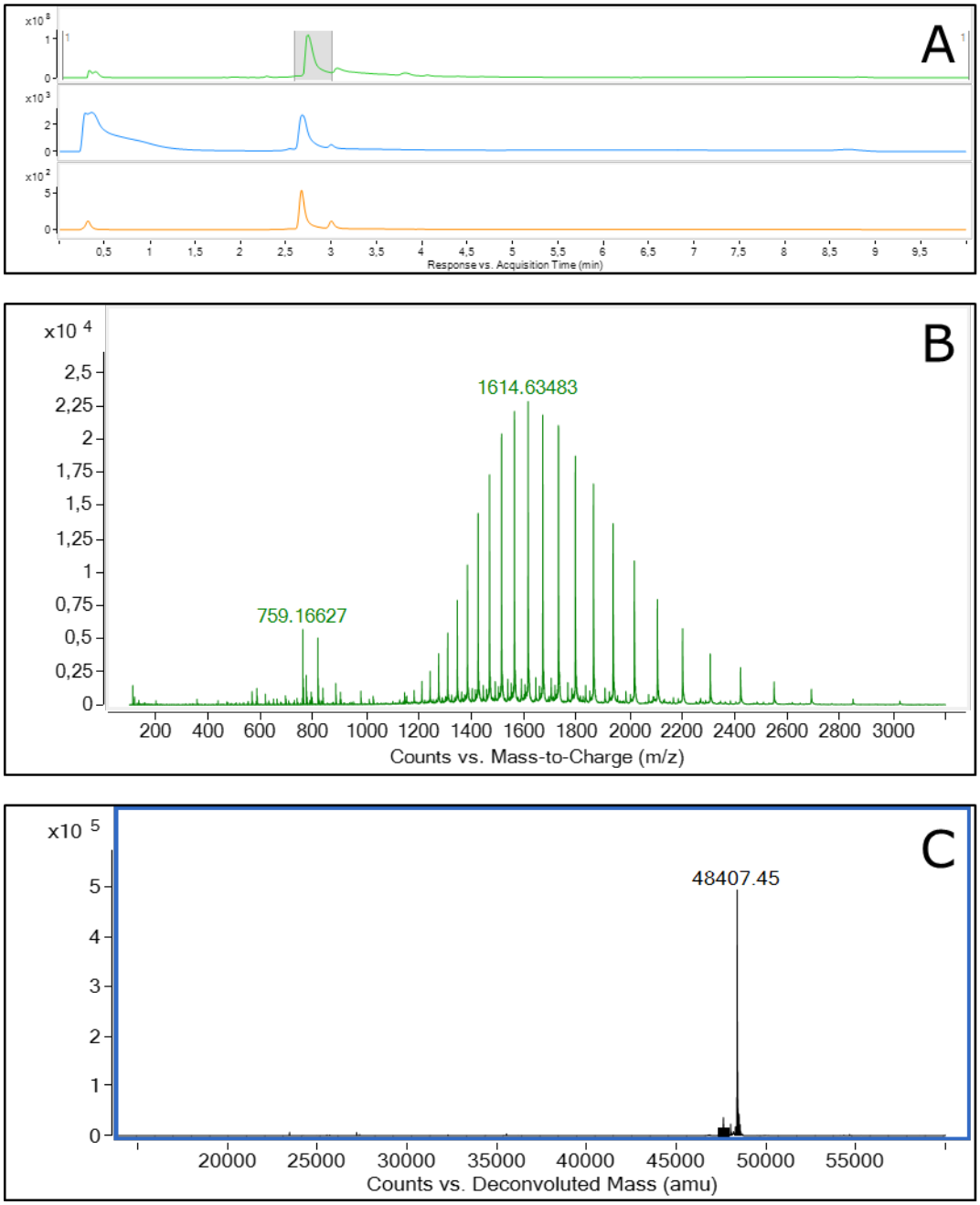


Figure S5. *The reaction between reduced Her-Fab and reagent* ***2*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 48407.45 corresponds to the Her-Fab rebridged with a single molecule of reagent* ***2*** *(Expected mass = 48407.53 Da).*

Her-Fab rebridged with reagent **3**

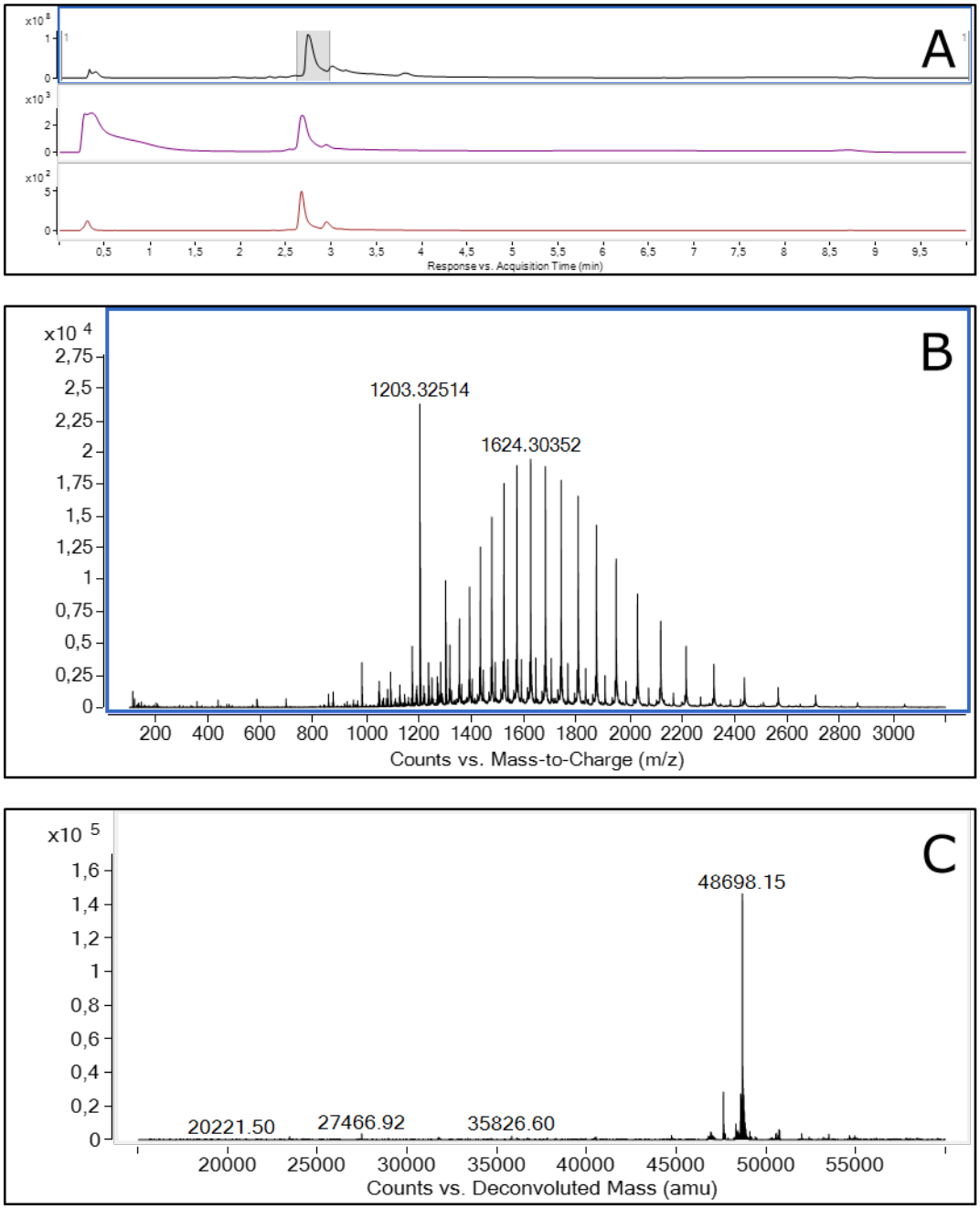


Figure S6. *The reaction between reduced Her-Fab and reagent **3** analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 48698.15 corresponds to the Her-Fab rebridged with a single molecule of reagent **3** (Expected mass = 48697.67 Da).*

Her-Fab reductive amination of reagent **1**

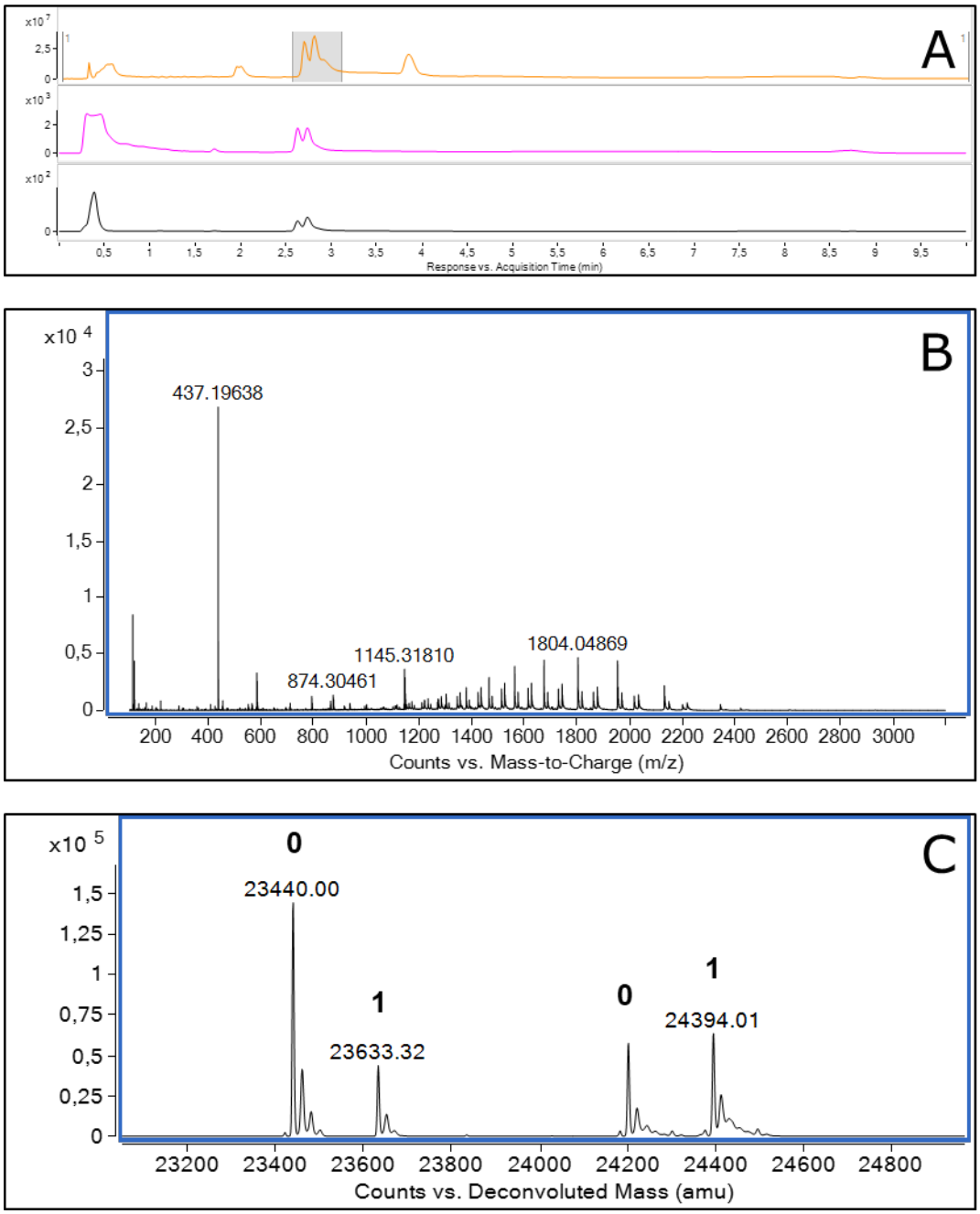


Figure S7. *The reduced reaction mixture following the reductive amination of rebridged Her-Fab and reagent* ***1*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. Light chain with 0 or 1 (Mass expected = 23634.06) thiol handle identified and heavy chain with 0 or 1 (Mass expected = 24394.20) fragment identified.*

Her-Fab reductive amination of reagent **2**

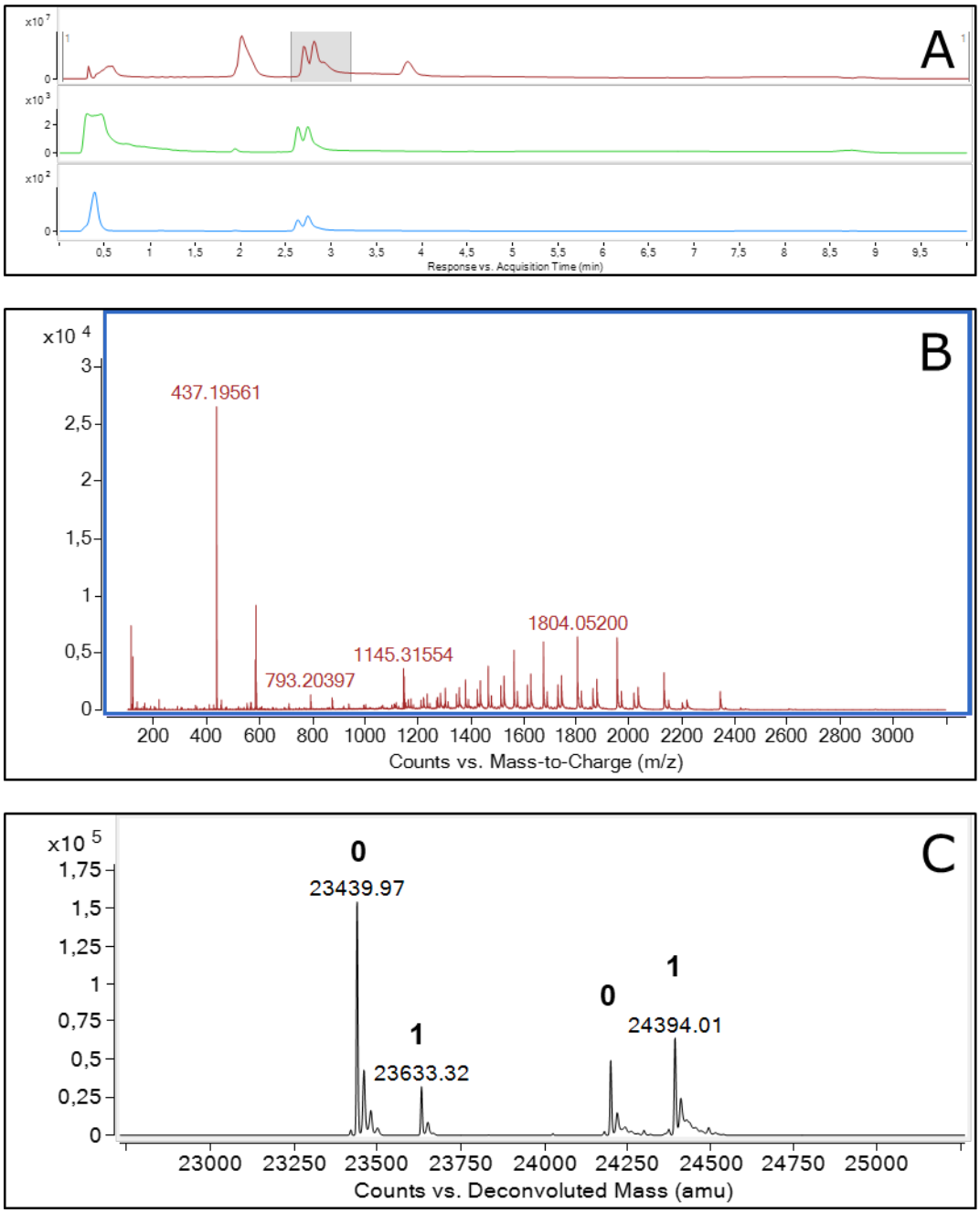


Figure S8. *The reduced reaction mixture following the reductive amination of rebridged Her-Fab and reagent* ***2*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. Light chain with 0 or 1 (Mass expected = 23634.06) thiol handle identified and heavy chain with 0 or 1 (Mass expected = 24394.20) fragment identified.*

Her-Fab reductive amination of reagent **3**

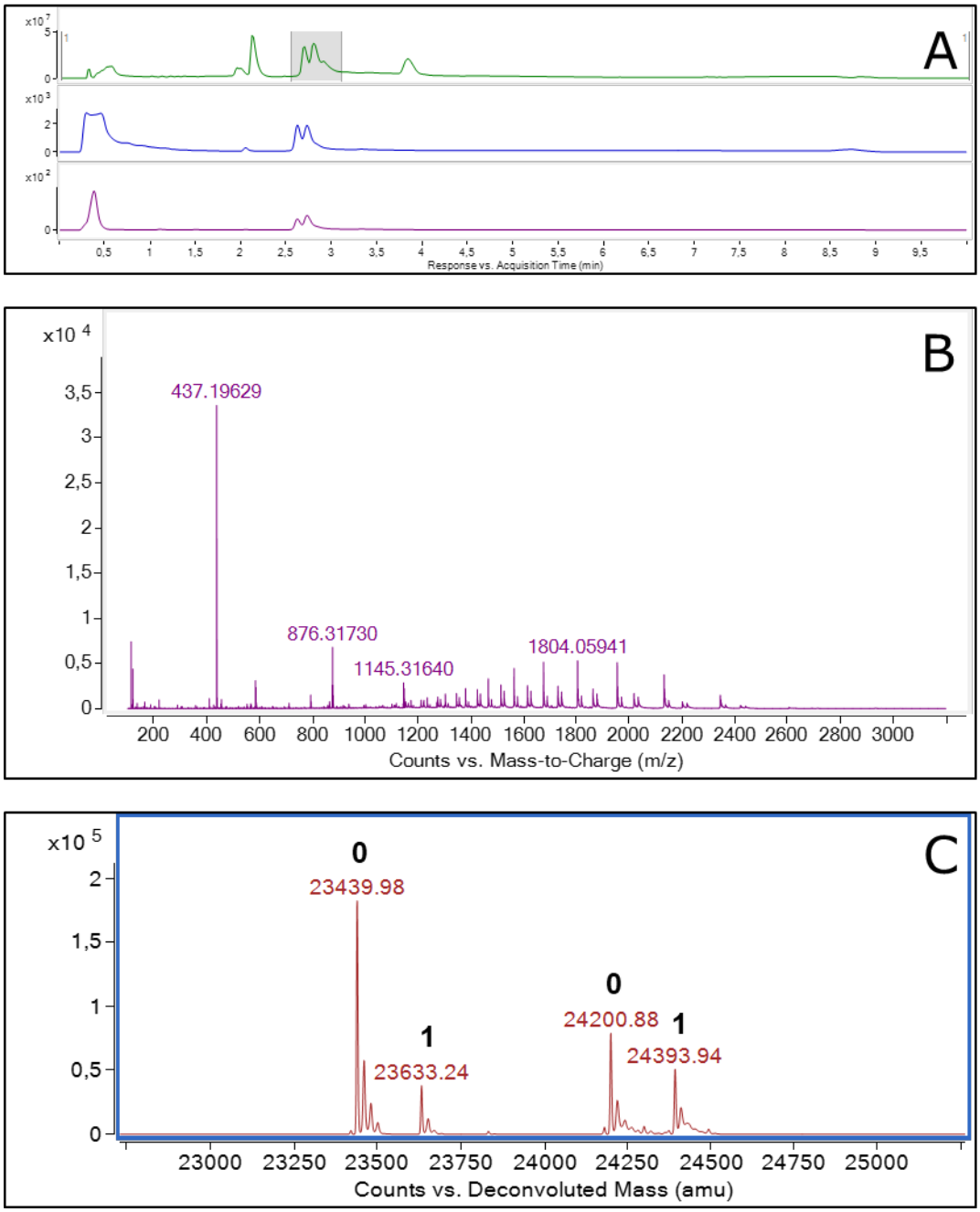


Figure S9. *The reduced reaction mixture following the reductive amination of rebridged Her-Fab and reagent* ***3*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. Light chain with 0 or 1 (Mass expected = 23634.06) thiol handle identified and heavy chain with 0 or 1 (Mass expected = 24394.20) fragment identified.*

Her-Fab reagent **1** conjugate NEM coupling

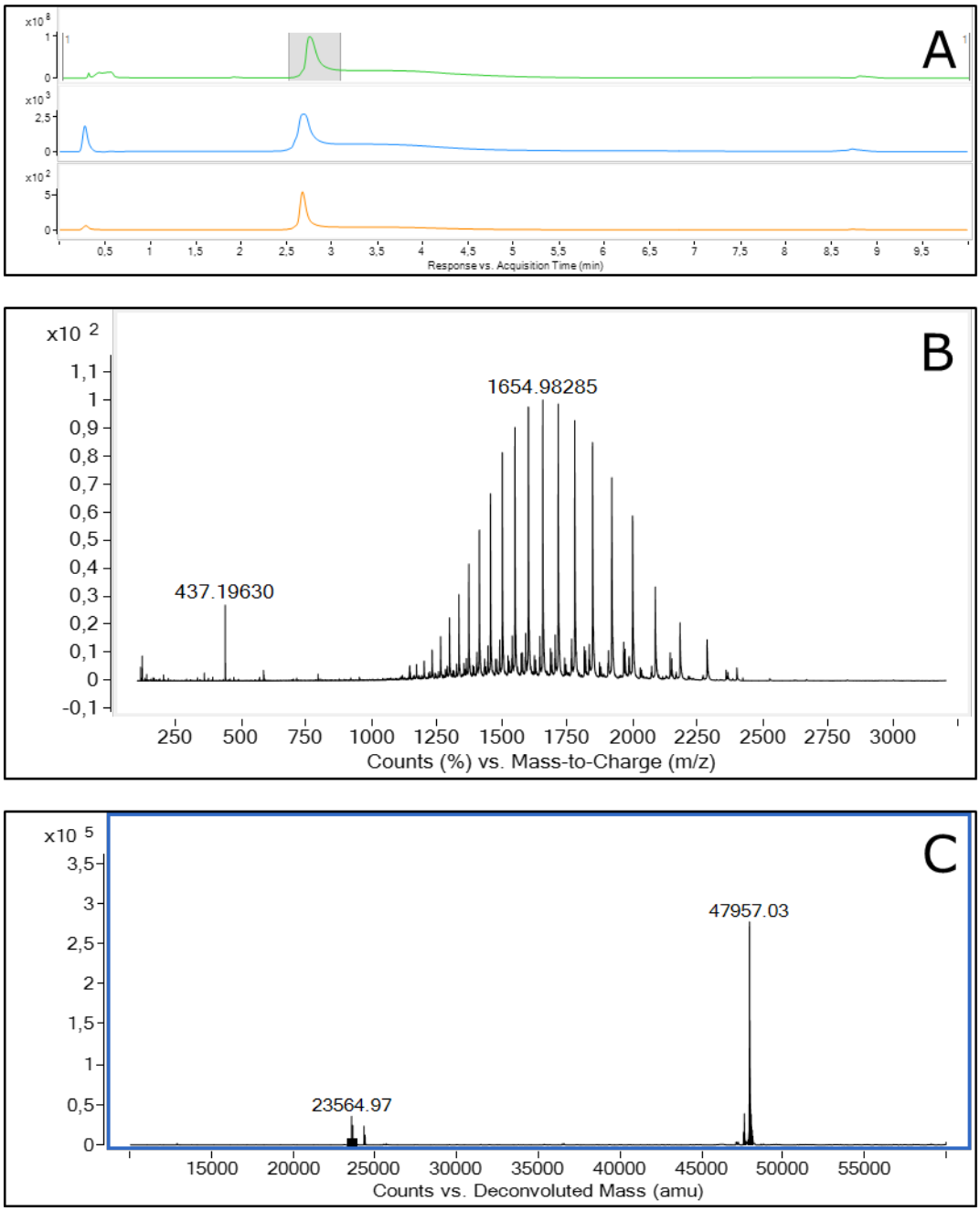


Figure S10. *The addition of NEM to the thiol handle, installed by reagent* ***1,*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 47957.03 corresponds to the addition of 1 molecule of NEM to the installed thiol handle. (Expected mass = 47957.43 Da)*

Her-Fab reagent **2** conjugate NEM coupling

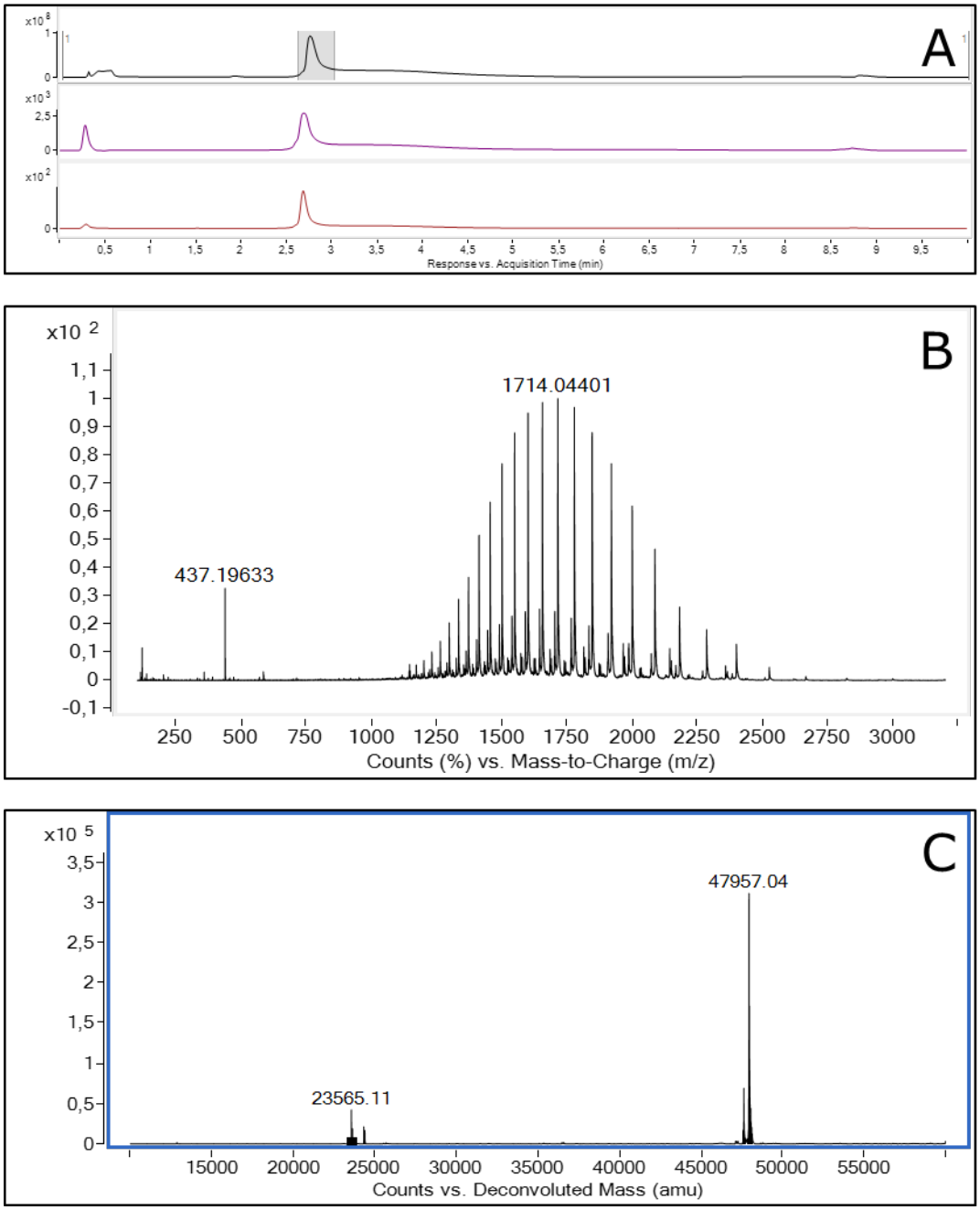


Figure S11. *The addition of NEM to the thiol handle, installed by reagent* ***2,*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 47957.04 corresponds to the addition of 1 molecule of NEM to the installed thiol handle. (Expected mass = 47957.43 Da)*

Her-Fab reagent **3** conjugate NEM coupling

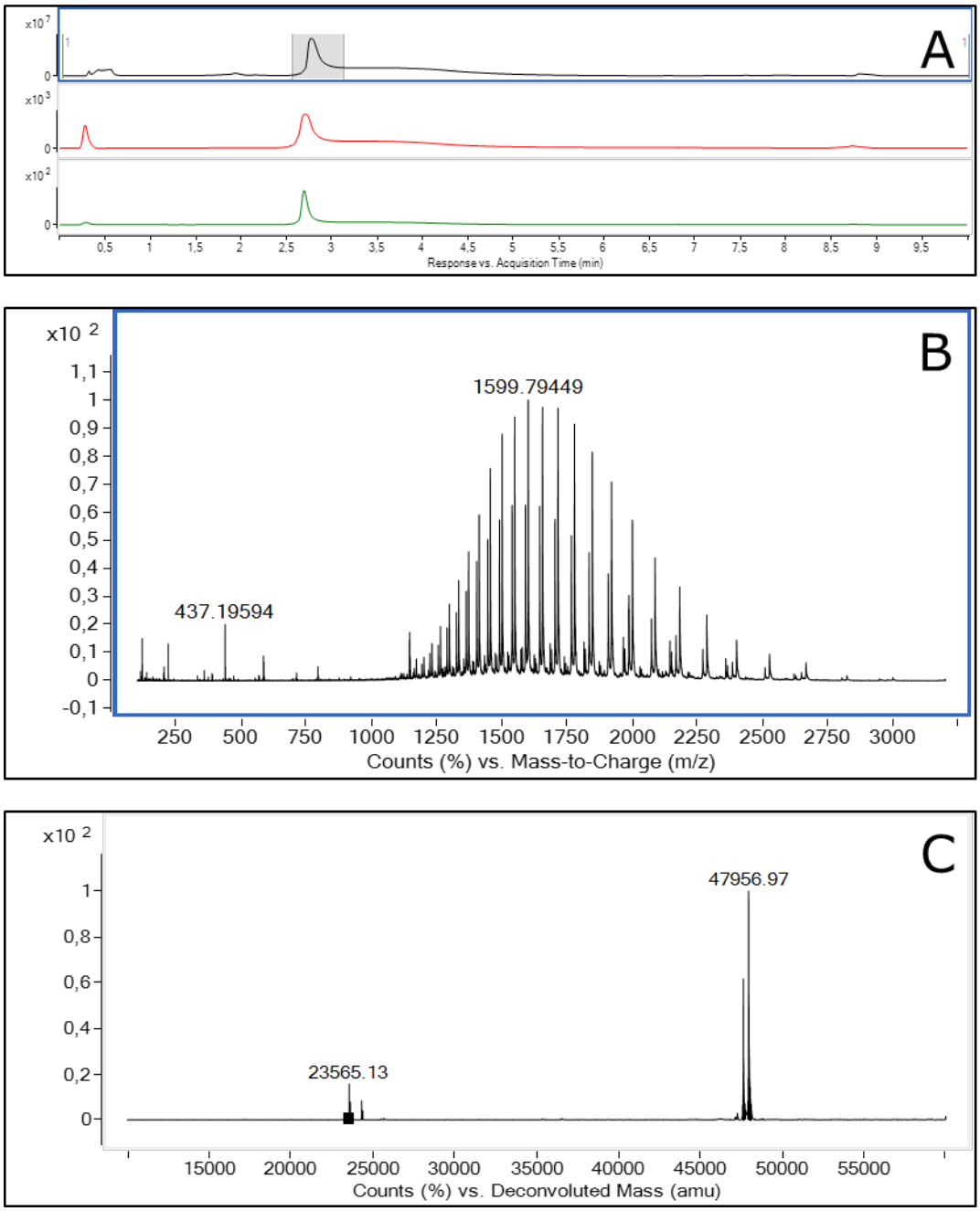


Figure S12. *The addition of NEM to the thiol handle, installed by reagent* ***3,*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 47956.97 corresponds to the addition of 1 molecule of NEM to the pseudo-cys handle. (Expected mass = 47957.43 Da)*

Her-Fab rebridged reaction time study with reagent **1**

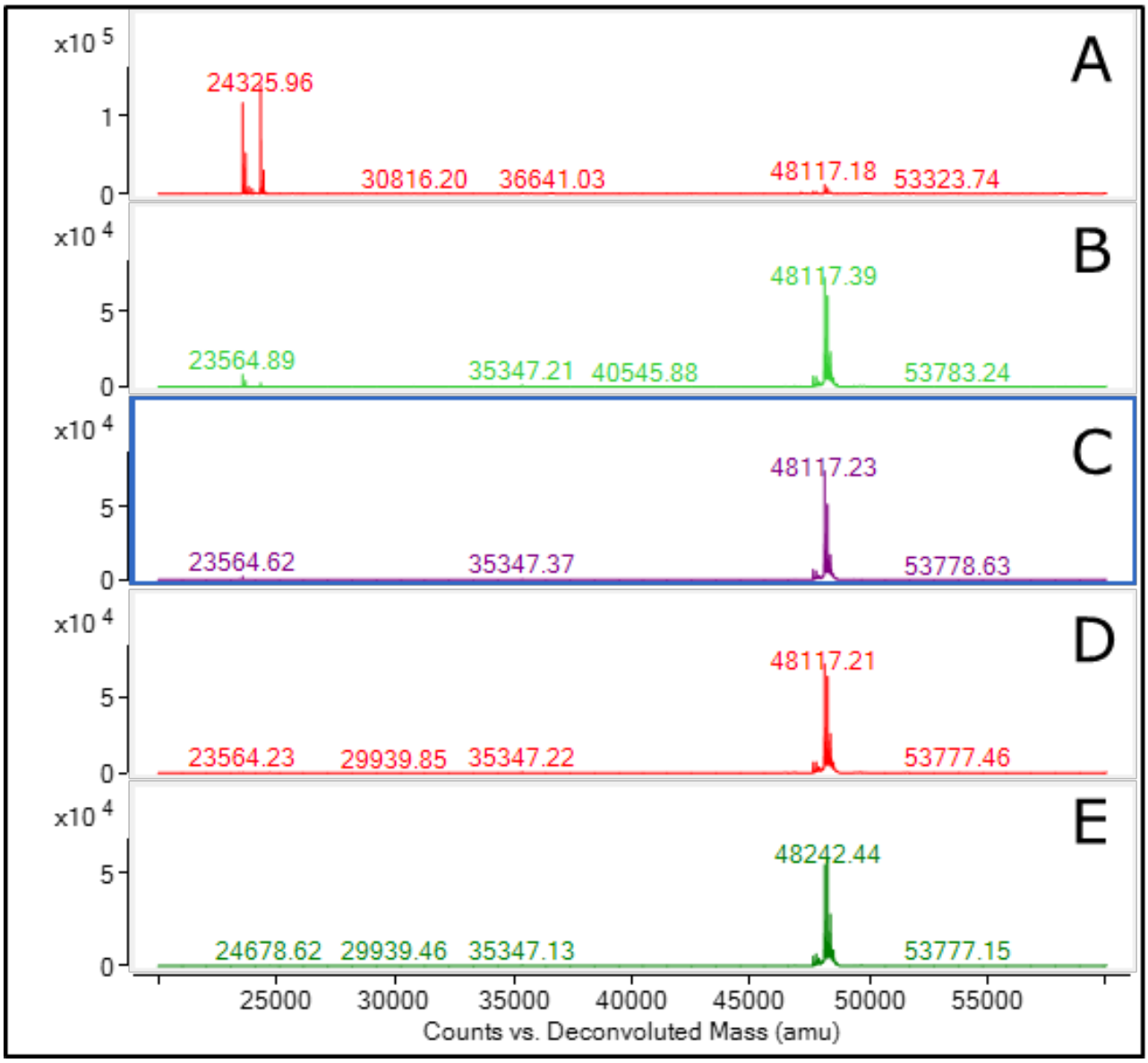


Figure S13. *The reaction between reduced Her-Fab and reagent* ***1*** *quenched with NEM at different time points, analysed by comparison of the ion intensities of the deconvoluted masses of the reduced and oxidised Fab from LCMS. Reduced Her-Fab (25 µM) was mixed with reagent* ***1*** *(75 µM). A sample was withdrawn and added to a vial containing 2000 eq. of NEM, which stopped the reaction at the following time points: A) directly after mixing - 0 minutes, B) 5 minutes, C) 10 minutes, D) 30 minutes, E) 60 minutes. The data suggests that 10 minutes is sufficient to nearly quantitatively staple the protein.*

Her-Fab screening of rebridged reaction - pH dependency

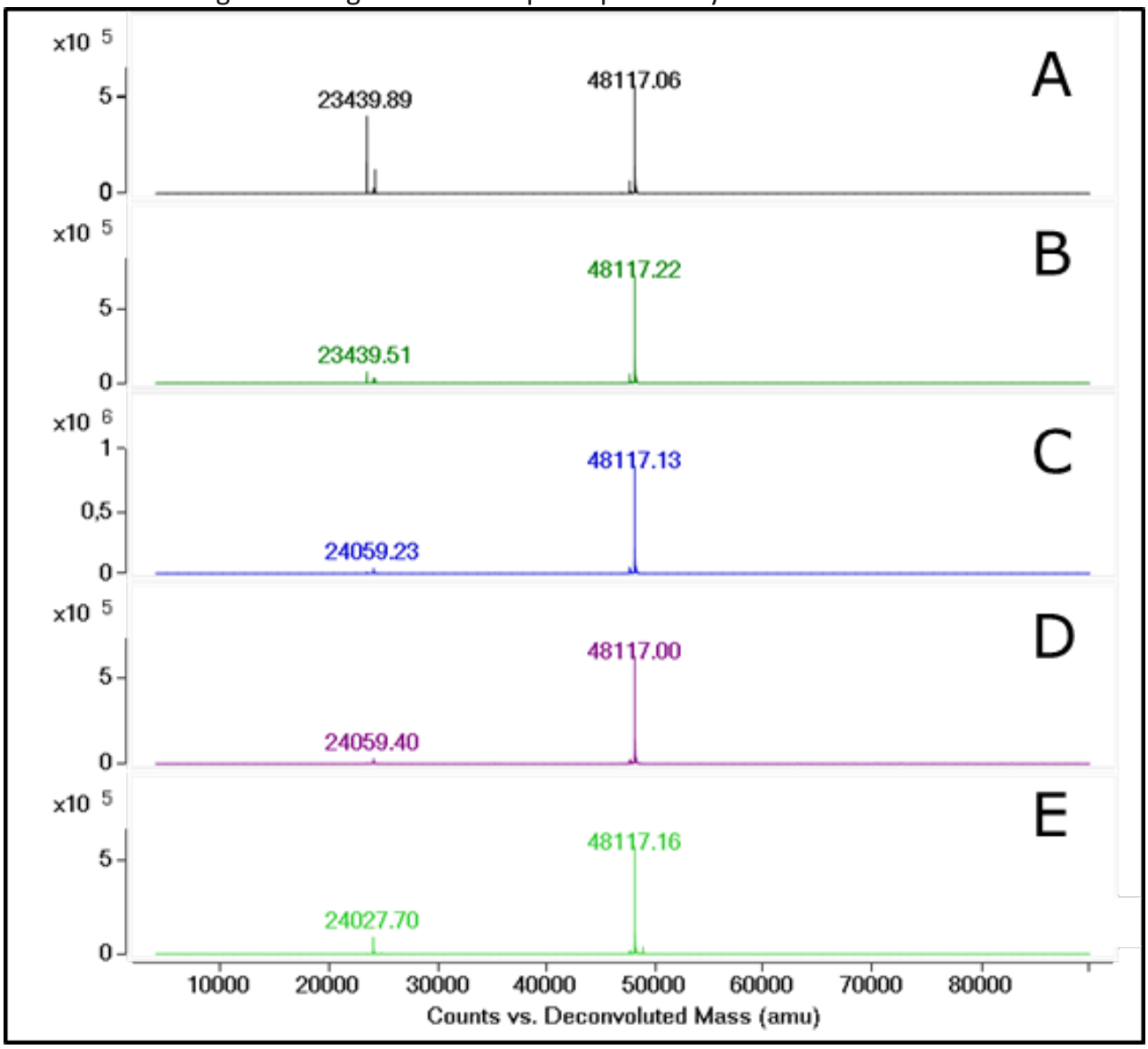


***Figure S14**. The reaction of Her-Fab with reagent **1** in various buffers adjusted to a range of pH values analysed by comparison of the ion intensities from LCMS of the deconvoluted masses of the reduced and oxidised Fab. The reaction between reagent and Fab starts to suffer at low pH and at higher pH the Fab starts to dissociate. The reactions were carried out in A) 50 mM MES buffer at pH= 5, B) 50 mM MES buffer at pH= 6, C) 0.1 M PBS buffer at pH= 7, D) 0.1 M PBS buffer at pH= 9, E) 0.1 M Na_2CO_3 buffer at pH = 10.8. The mass of 24027.70 corresponds to reagent added with one intact activated disulphide.*

Her-Fab appended with other small molecule maleimides

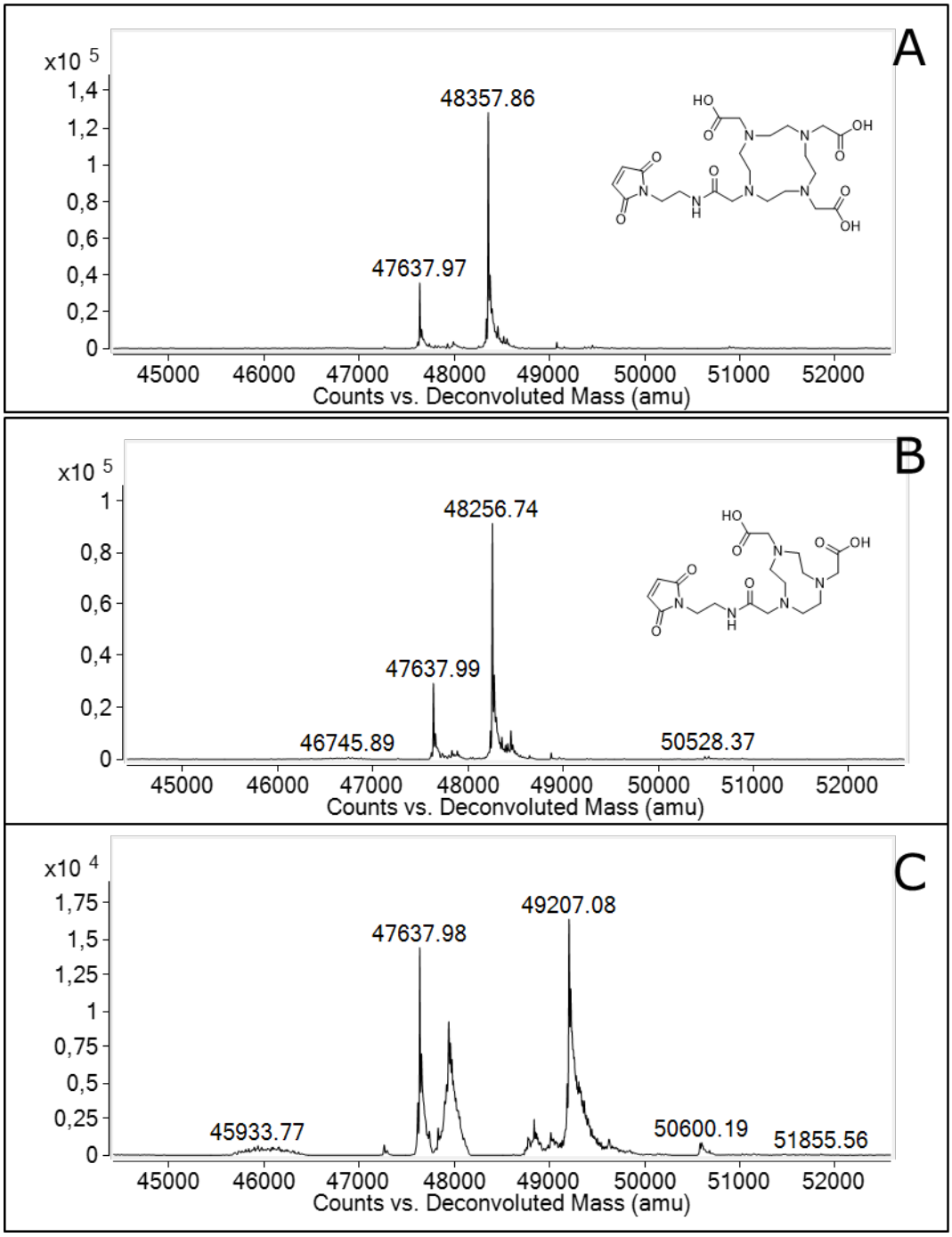


Figure S15. *The conjugation of other commercially available small molecule maleimides to a pseudo cysteine handle on Her-Fab. A) addition of DOTA-maleimide (structure shown in A). Expected mass with 1 addition of DOTA = 48358.62 Da. B) Addition of NOTA-maleimide (structure shown in B). Expected mass with 1 addition of NOTA = 48257.58 Da. C) Addition of Vivotag™680XL. The structure is unknown, but the 49207 Da is expected to be the conjugate with 1 vivotag. A large peak +305 Da was seen for the vivotag addition – this could be a breakdown product of the fluorophore formed during LCMS.*

Ava-Fab experiments

Ava-Fab native

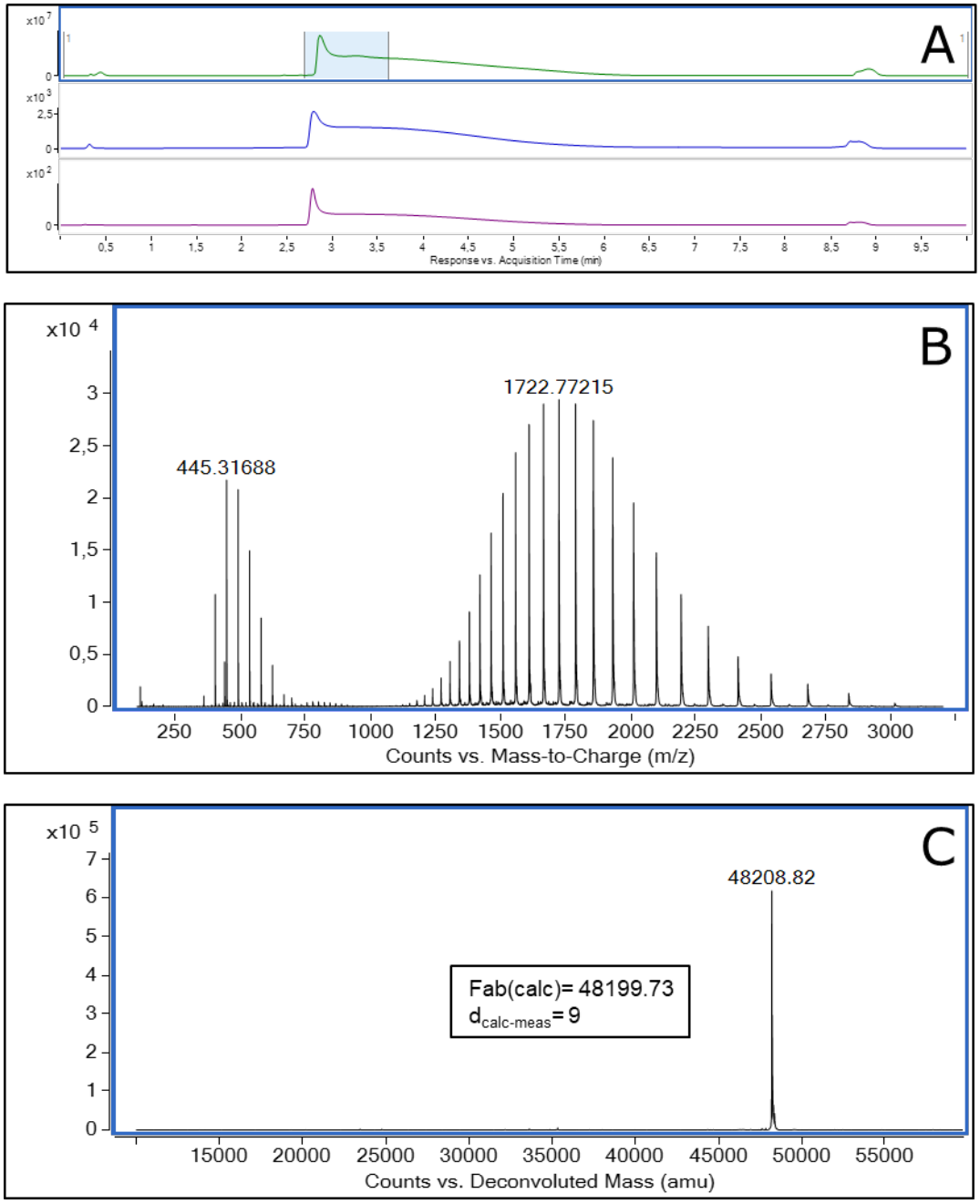


Figure S16. *The ÄKTA-purified Ava-Fab following papain digestion of Avastin® analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B.*

Ava-Fab reduced

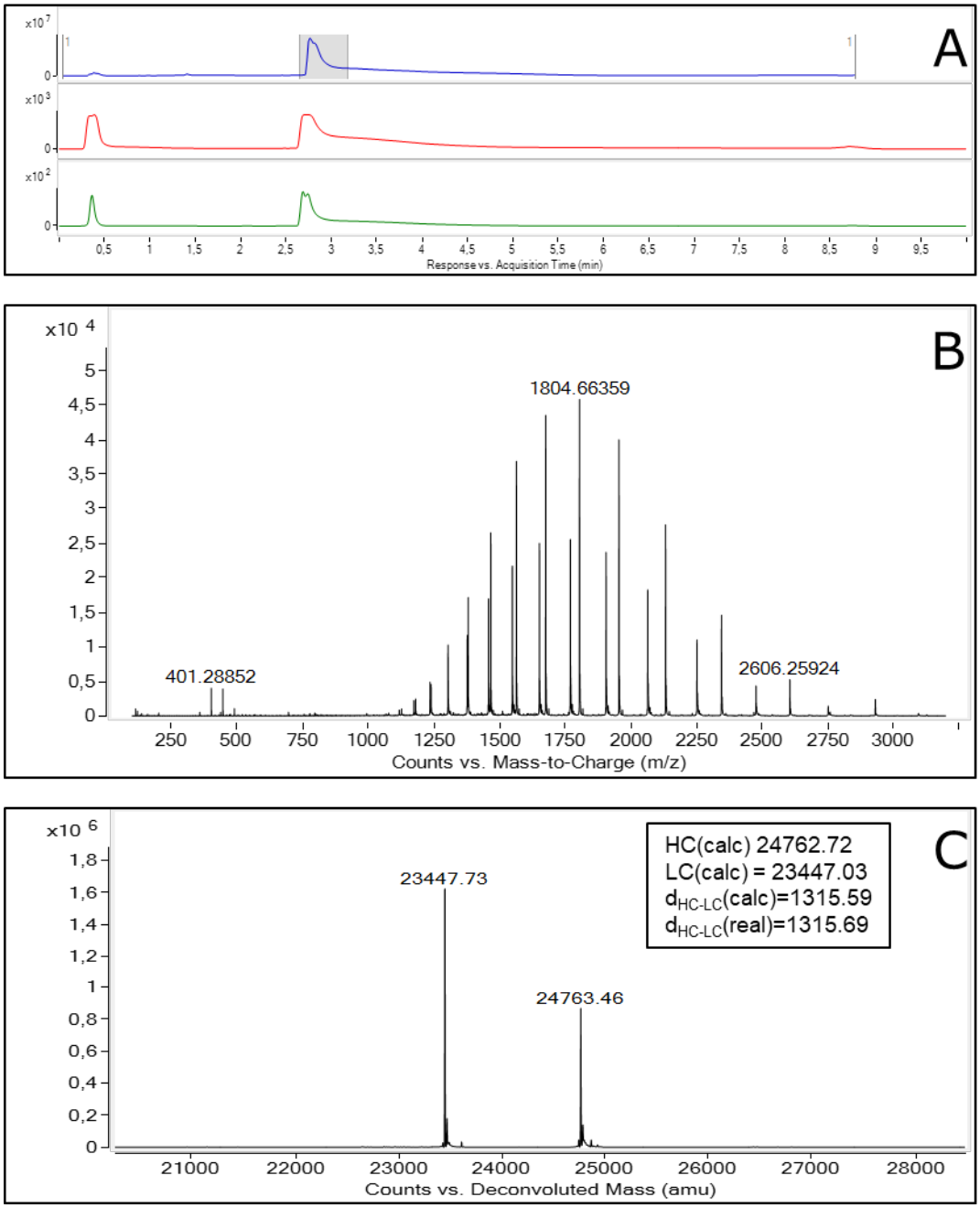


Figure S17. *The reduction of Ava-Fab with DTT analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses of light chain and heavy chain.*

Ava-Fab rebridged reaction reagent **1**

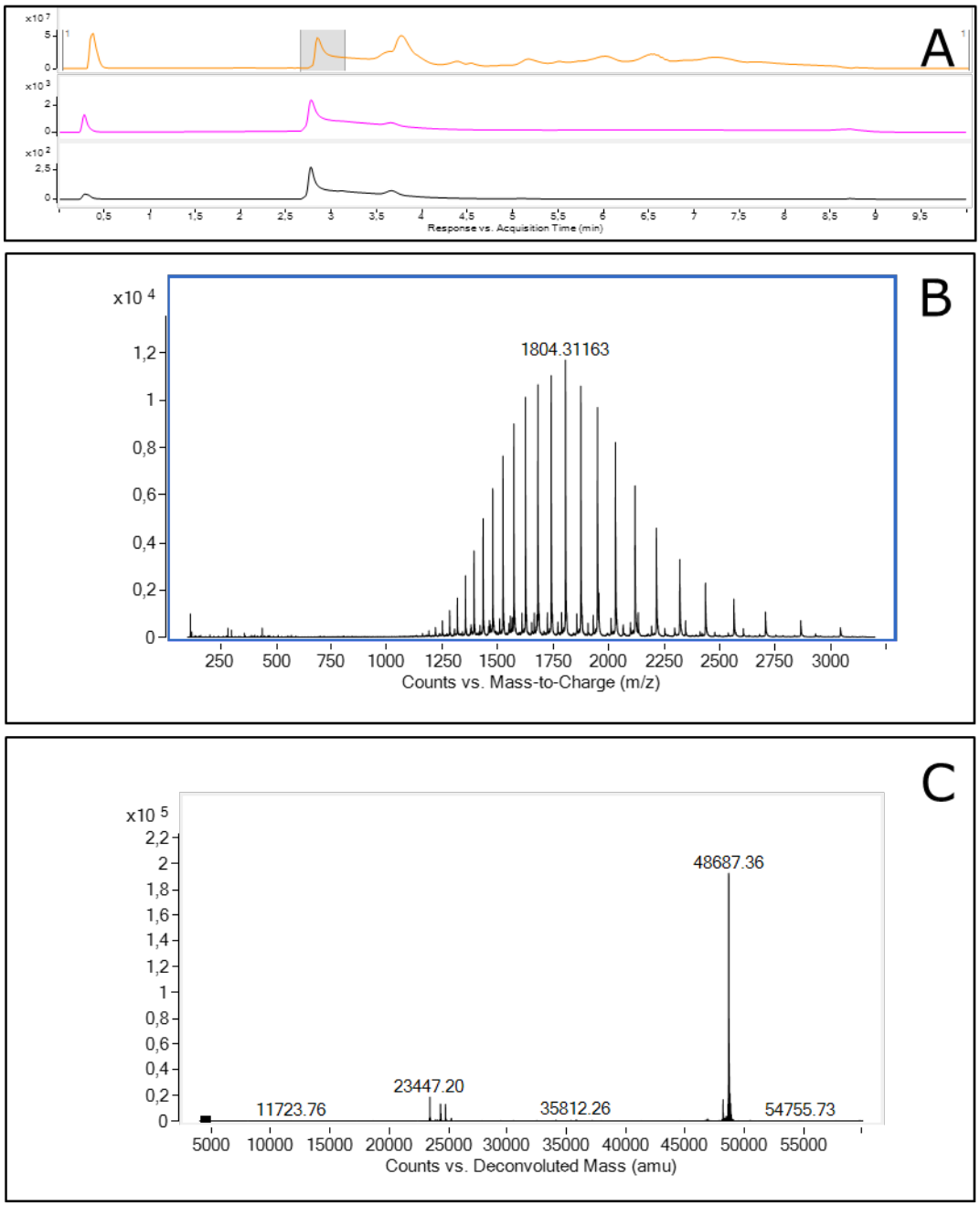


Figure S18. *The rebridged reaction between reduced Ava-Fab and reagent* ***1*** *analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 48687.36 corresponds to the addition of 1 reagent molecule to the Fab. (Expected mass = 48687.88 Da)*

Ava-Fab NEM conjugation reagent **1**

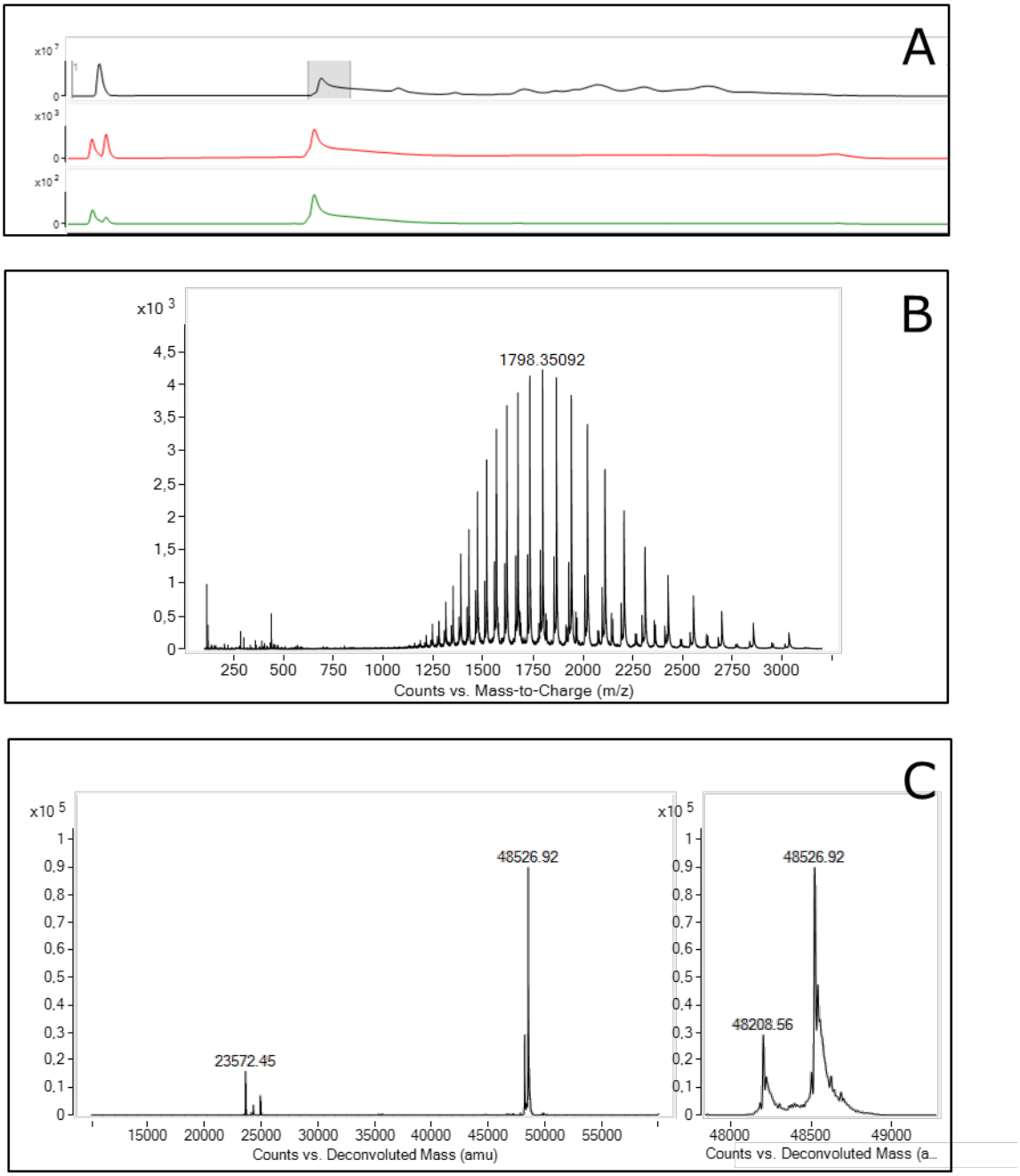


Figure S19. *The reaction of the Ava-Fab pseudo-cys handle installed by reagent **1** with NEM analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 48526.92 corresponds to the addition of 1 molecule of NEM to the pseudo-cys handle. (Expected mass = 48527.93 Da)*

Ben-Fab

Ben-Fab native Fab

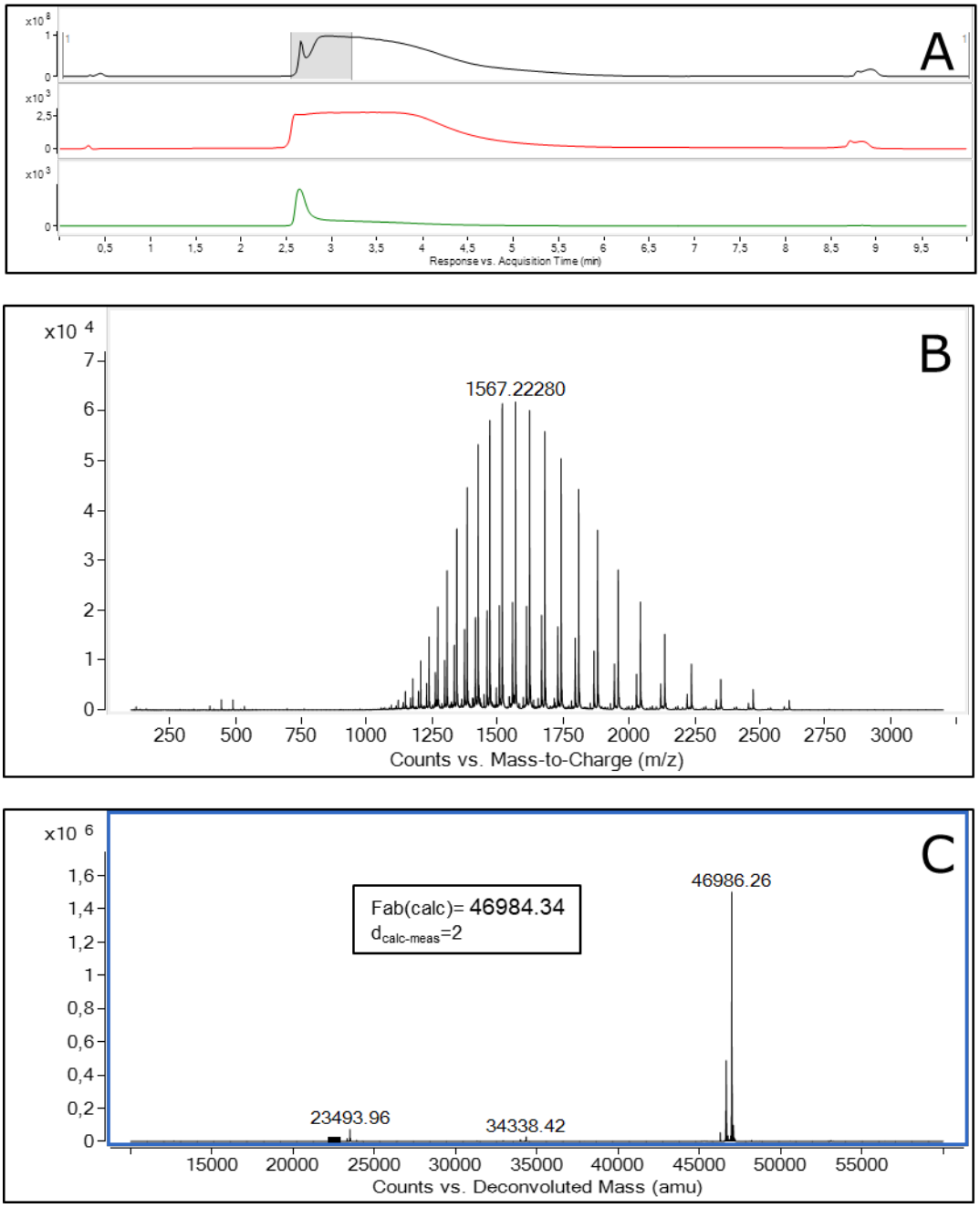


Figure S20. *The ÄKTA-purified Ben-Fab following papain digestion of Benlysta®. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box B) Counts vs. m/z of scan. C) Deconvolution of B. The Fab contains an impurity or truncated product that was not successfully removed.*

Ben-Fab reduced

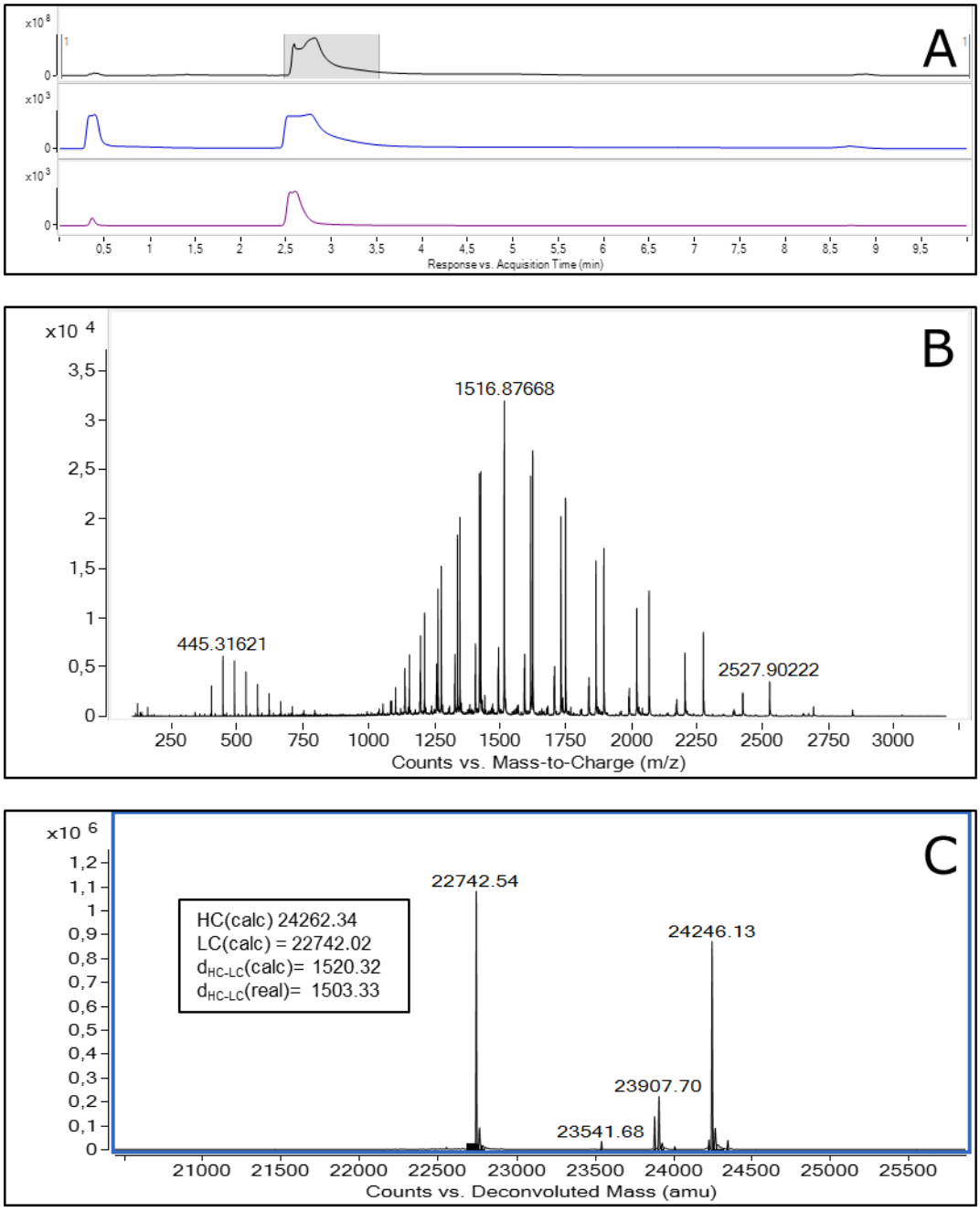


Figure S21. *Ben-Fab reduced with DTT and analysed by LC-MS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the (grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses of light chain and heavy chain.*

Ben-Fab rebridged reaction reagent **1**

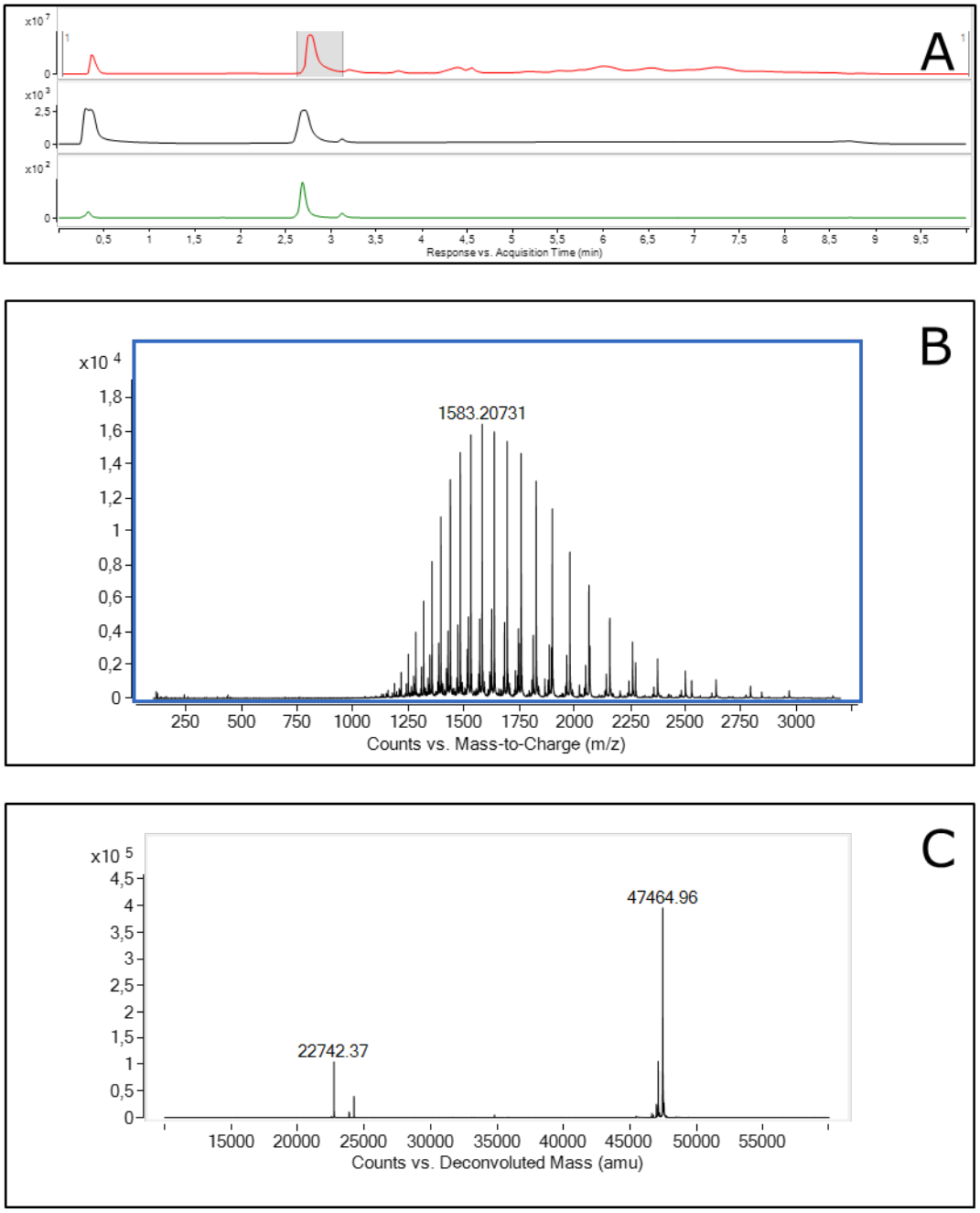


Figure S22. *The rebridged reaction between reduced Ben-Fab and reagent **1** analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 47464.96 corresponds to the rebridging of the Fab with one reagent **1** molecule to the Fab. (Expected mass = 47465.32 Da)*

Ben-Fab NEM conjugation reagent **1**

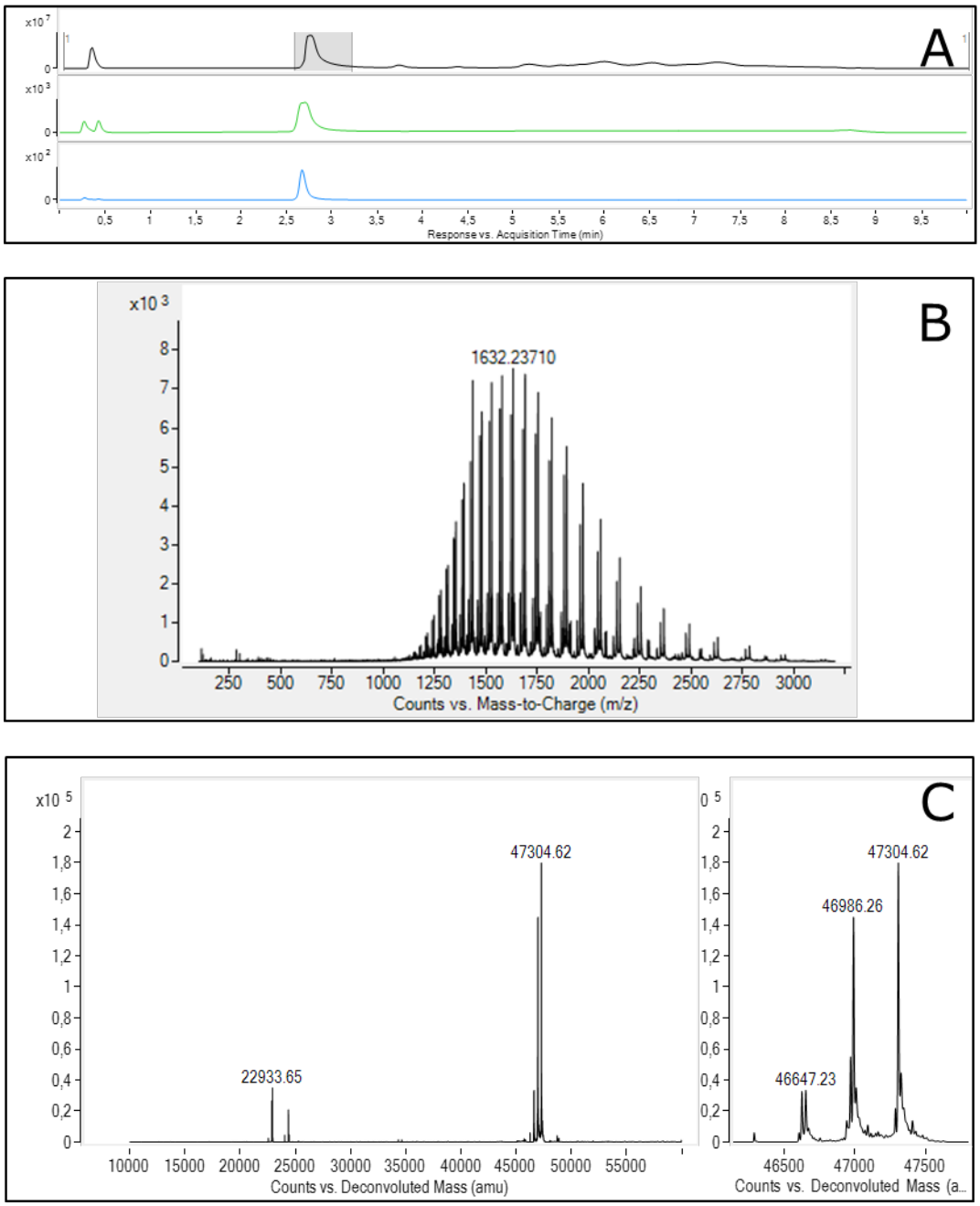


Figure S23. *The reaction of a Ben-Fab pseudo-cys handle installed by reagent **1** with NEM analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey area in A). C) Deconvolution of B. The mass of 47304.62 corresponds to the addition of 1 molecule of NEM to the pseudo-cys handle. (Expected mass = 47305.37 Da)*

Erb-Fab

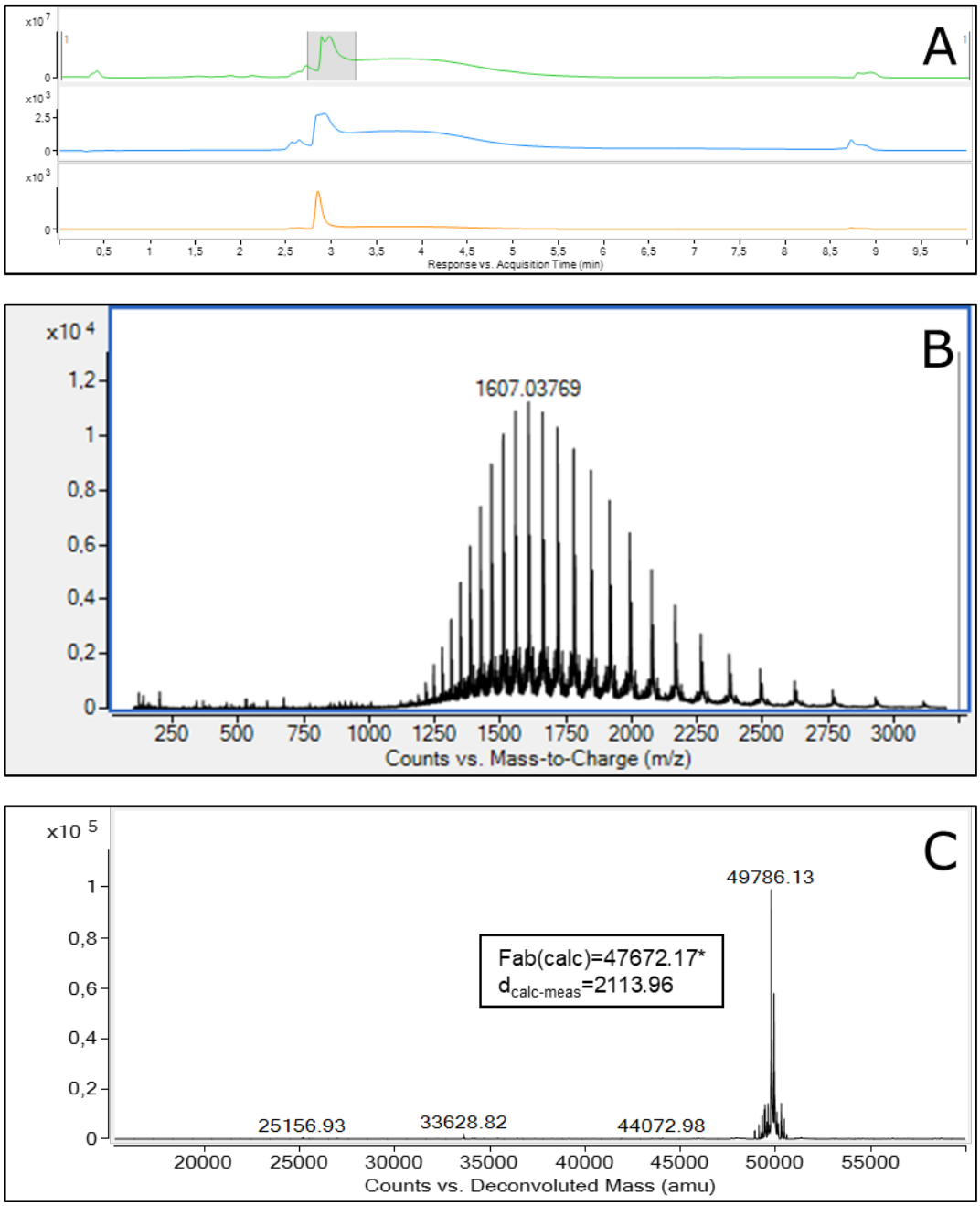


Figure S24. *The ÄKTA-purified Erb-Fab following papain digestion of Erbitux®. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom. The, scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B. The erb-Fab is non-homogenously glycosylated on N88 of the heavy chain, which leads to a higher than expected mass and splitting of the peak.*

Erb-Fab reduced

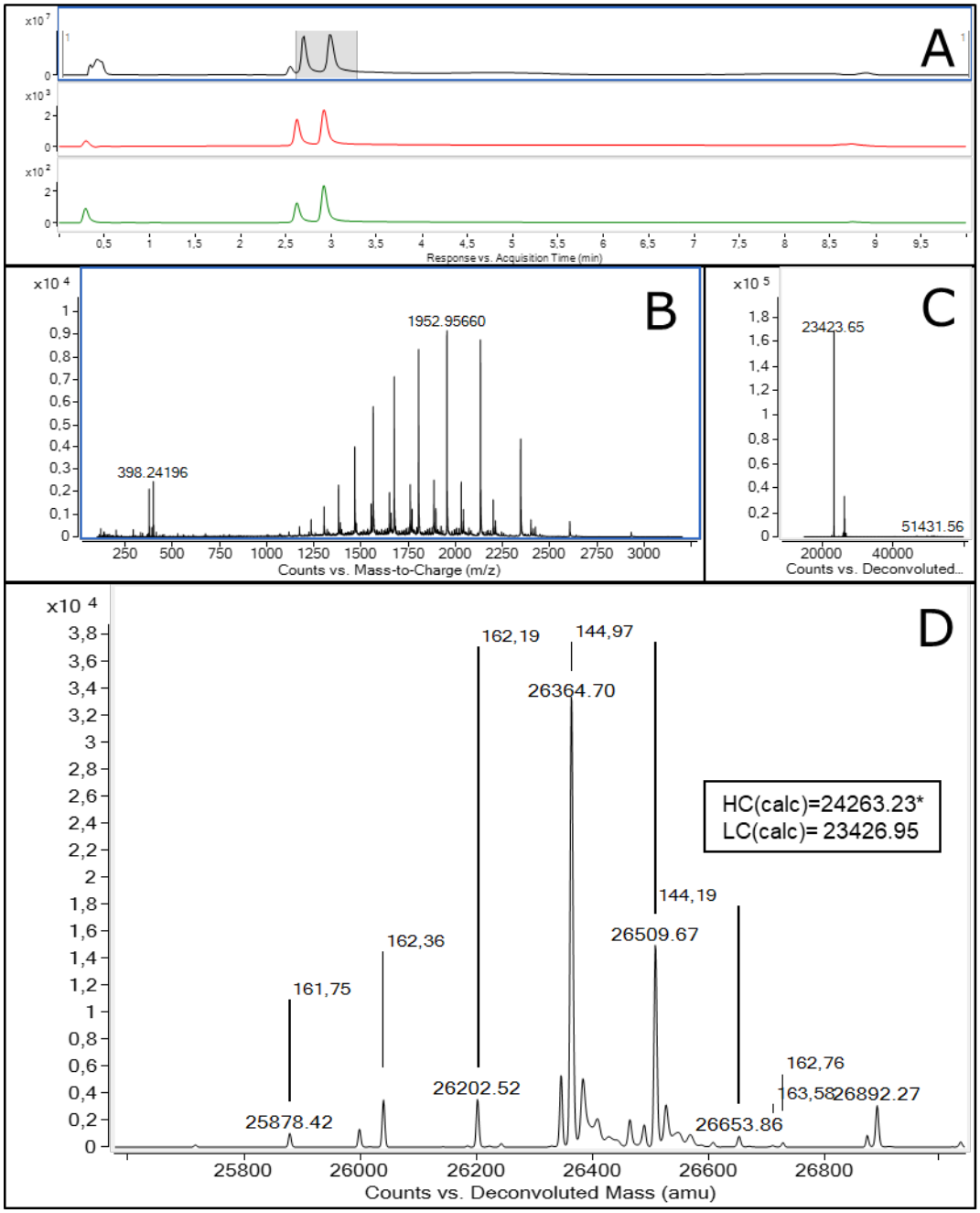


Figure S25. *The reduction of Erb-Fab with DTT analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses of light chain and heavy chain. Due to the variation in the glycan on the N88 of the HC, the peak for the full Fab and for the HC are split in multiple peaks with either a fucose (145 Da) or hexose (162 Da) between them.*

Erb-Fab rebridged reaction reagent **1**

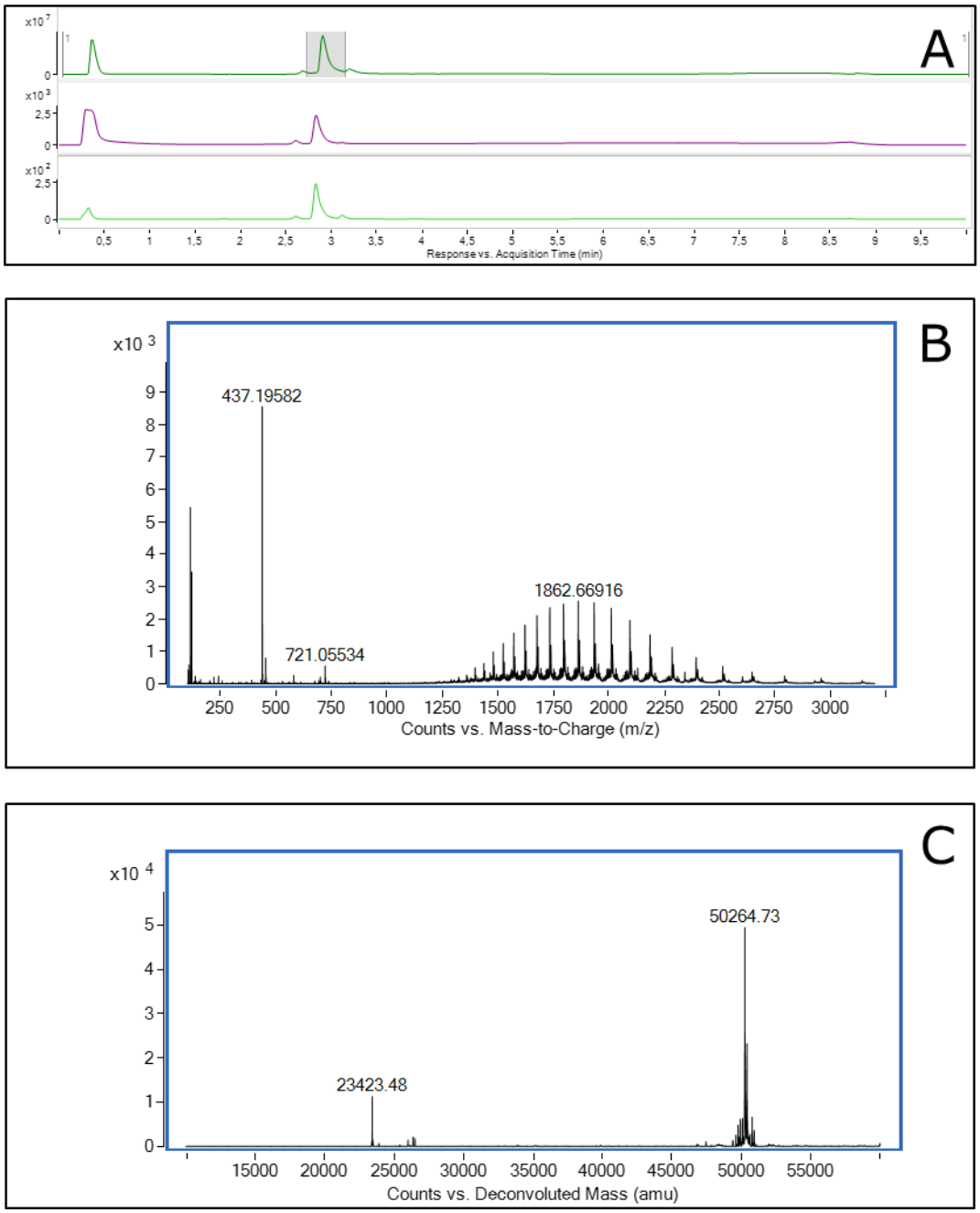


Figure S26. *The rebridged reaction between reduced Erb-Fab and reagent* ***1*** *analysed by LCMS.* ***A)*** *TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom) B) m/z of the scan range indicated by the grey box in A). C) Deconvolution of B. The mass of 50264.73 corresponds to the rebridging of the Fab with one reagent* ***1*** *molecule. (Expected mass = 50265.19 Da.)*

Erb-Fab NEM conjugation reagent **1**

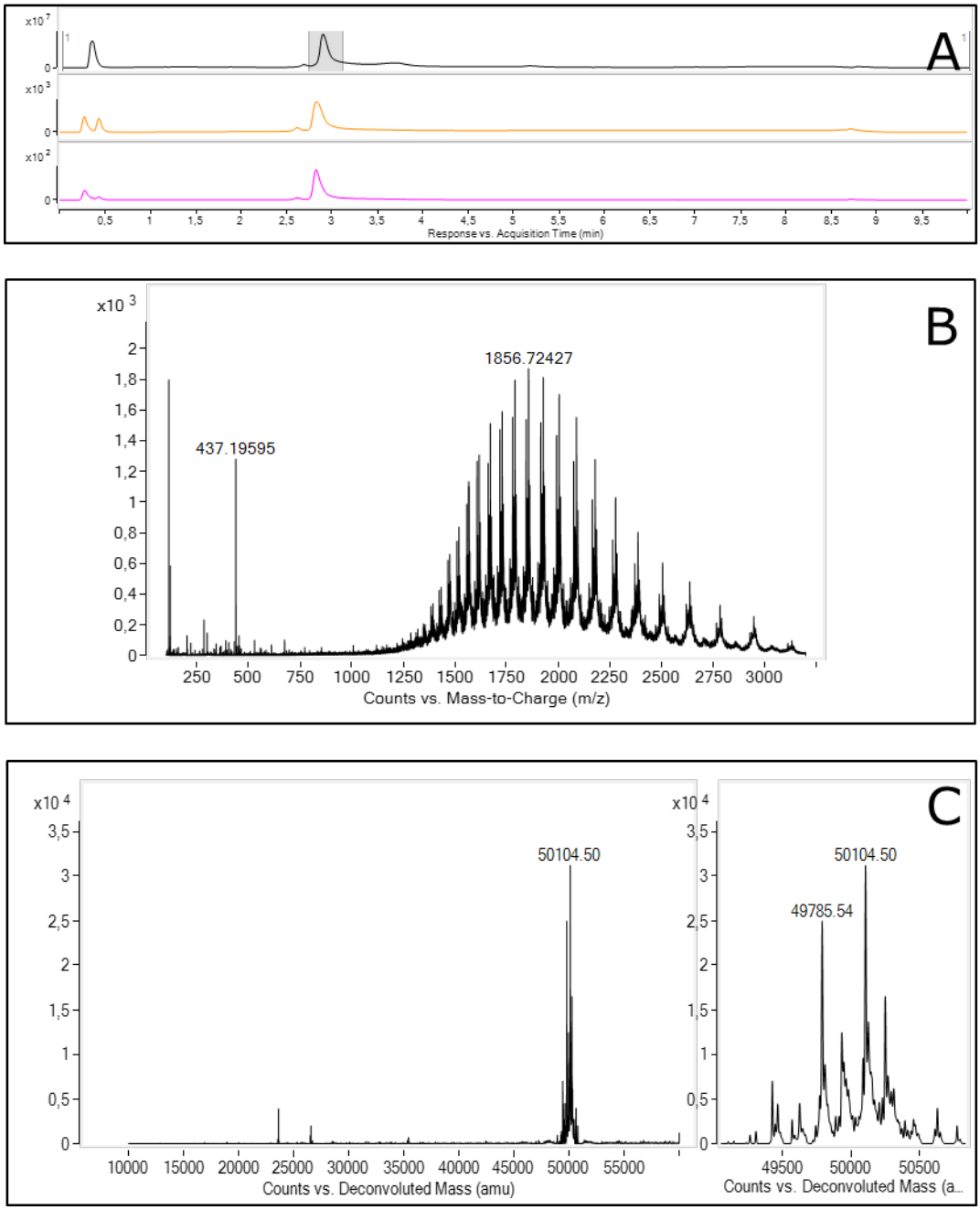


Figure S27. *The NEM-conjugation to pseudo-cysteine Erb-Fab analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses. Due to the variable glycans on the HC, the peak for the full Fab and for the HC are split in multiple peaks with either a fucose (145 Da) or hexose (162 Da) between them. The expected +318 Da is observed between the masses 49785.54 Da and 50105.5 Da.*

Rem-Fab experiments

Rem-Fab native

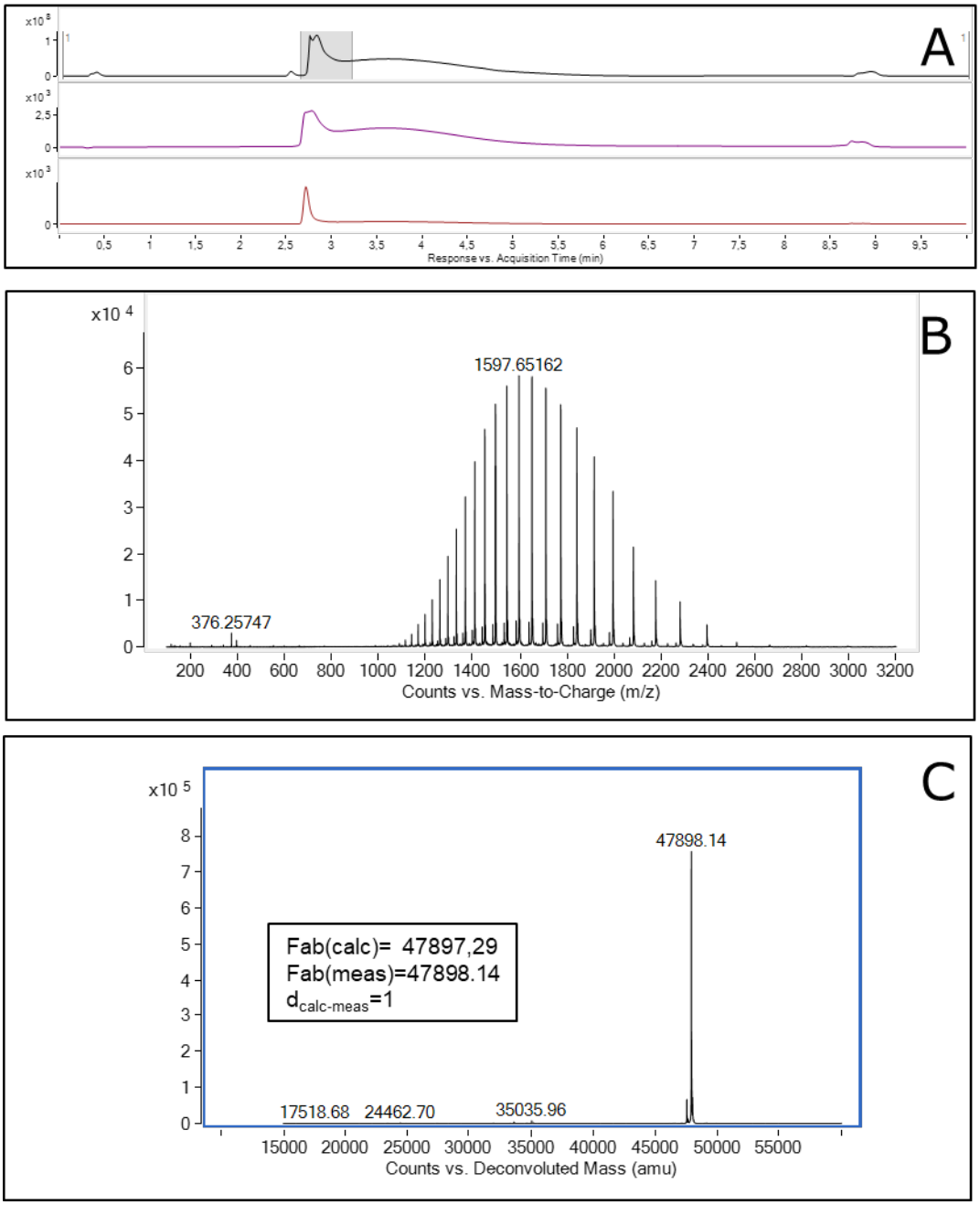


Figure S28. *The ÄKTA-purified Rem-Fab following papain digestion of Remicade® analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan range. C) Deconvolution of B.*

Rem-Fab reduced

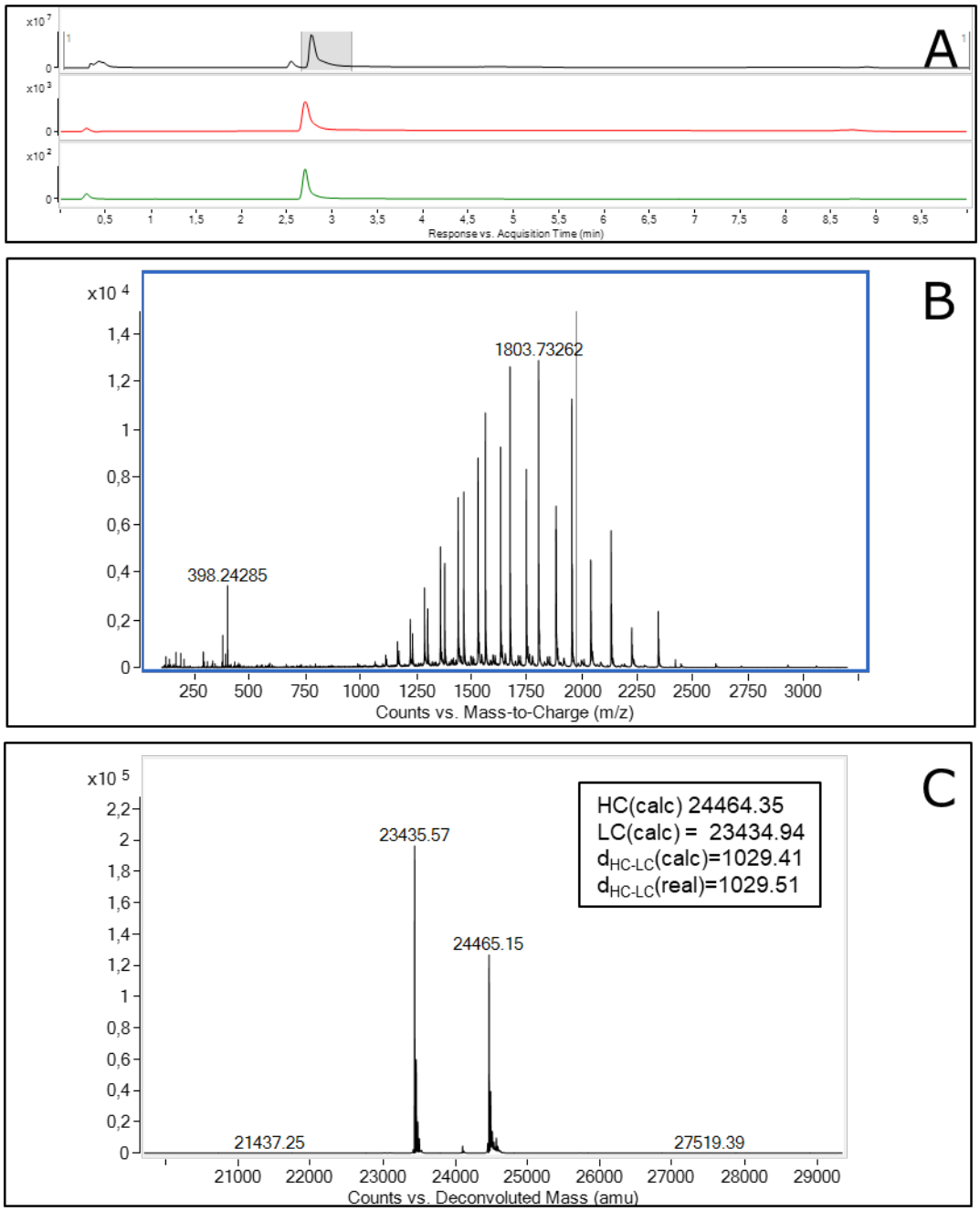


Figure S29. *The reduction of Rem-Fab with DTT analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses of light chain and heavy chain.*

Rem-Fab rebridged with reagent **1**

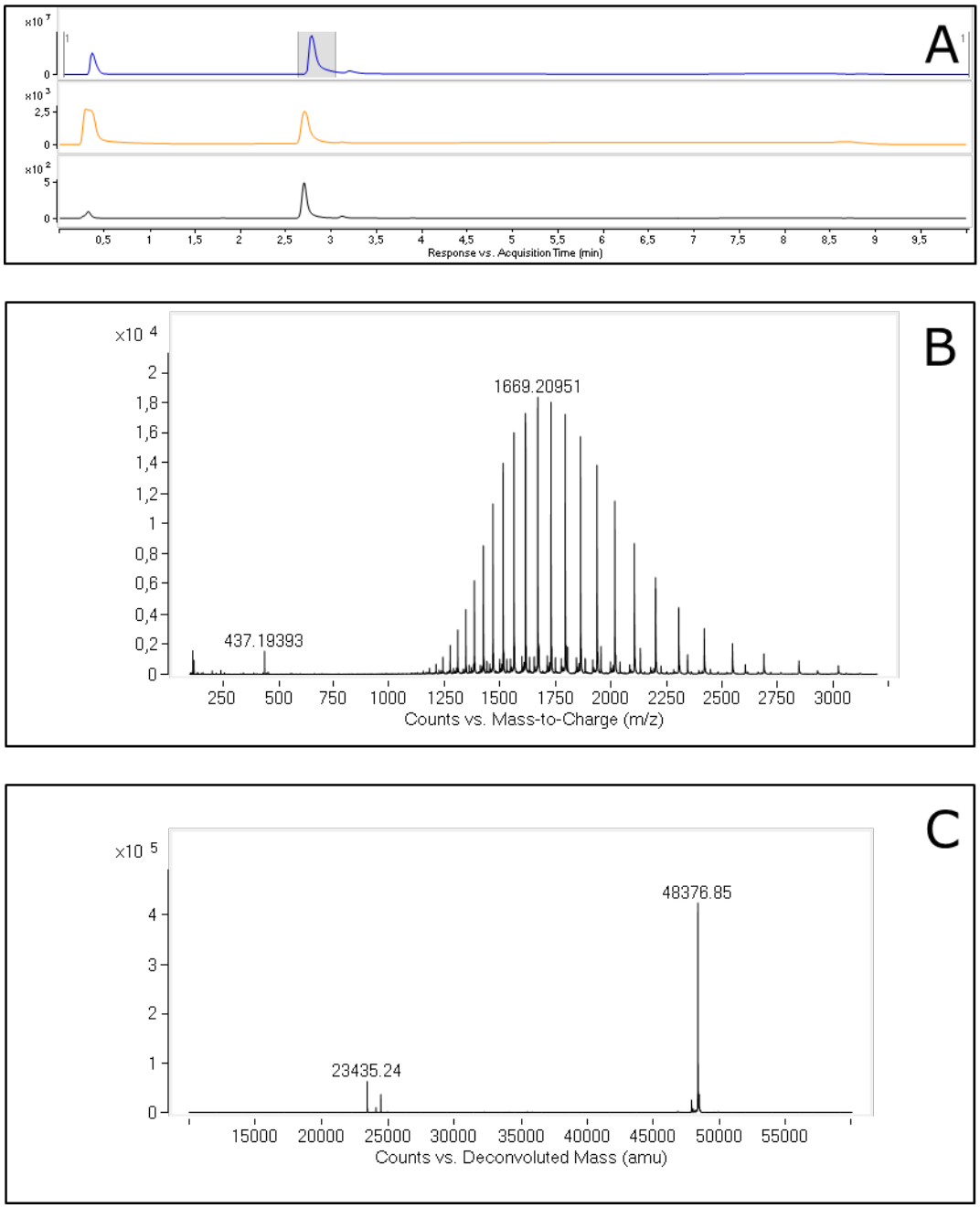


Figure S30. *The rebridged reaction between the reduced Rem-Fab and reagent **1** analysed by LCMS. A) TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). B) m/z of the scan range (grey box in A). C) Deconvolution of B. The mass of 48376.85 corresponds to the rebridging with a single reagent **1** molecule to the Fab. (Expected mass = 48376.35 Da).*

Rem-Fab NEM conjugate

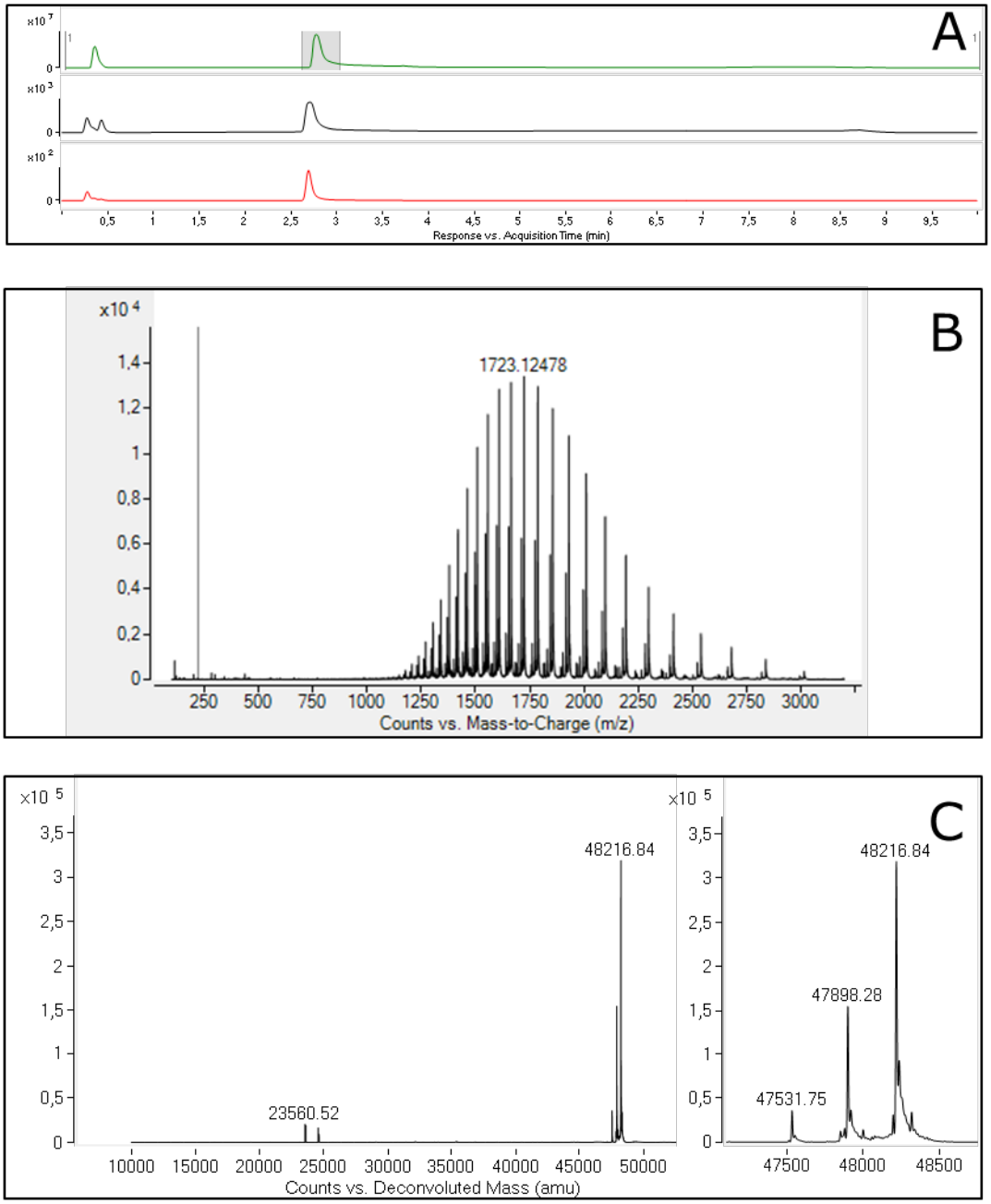


Figure S31. *The NEM-conjugation to pseudo-cysteine Rem-Fab analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses. The mass of 48216.84 corresponds to the addition of 1 molecule of NEM to the handle. (Expected mass = 48216.40 Da.)*

Rem-Fab rebridged reaction time study with reagent **1**

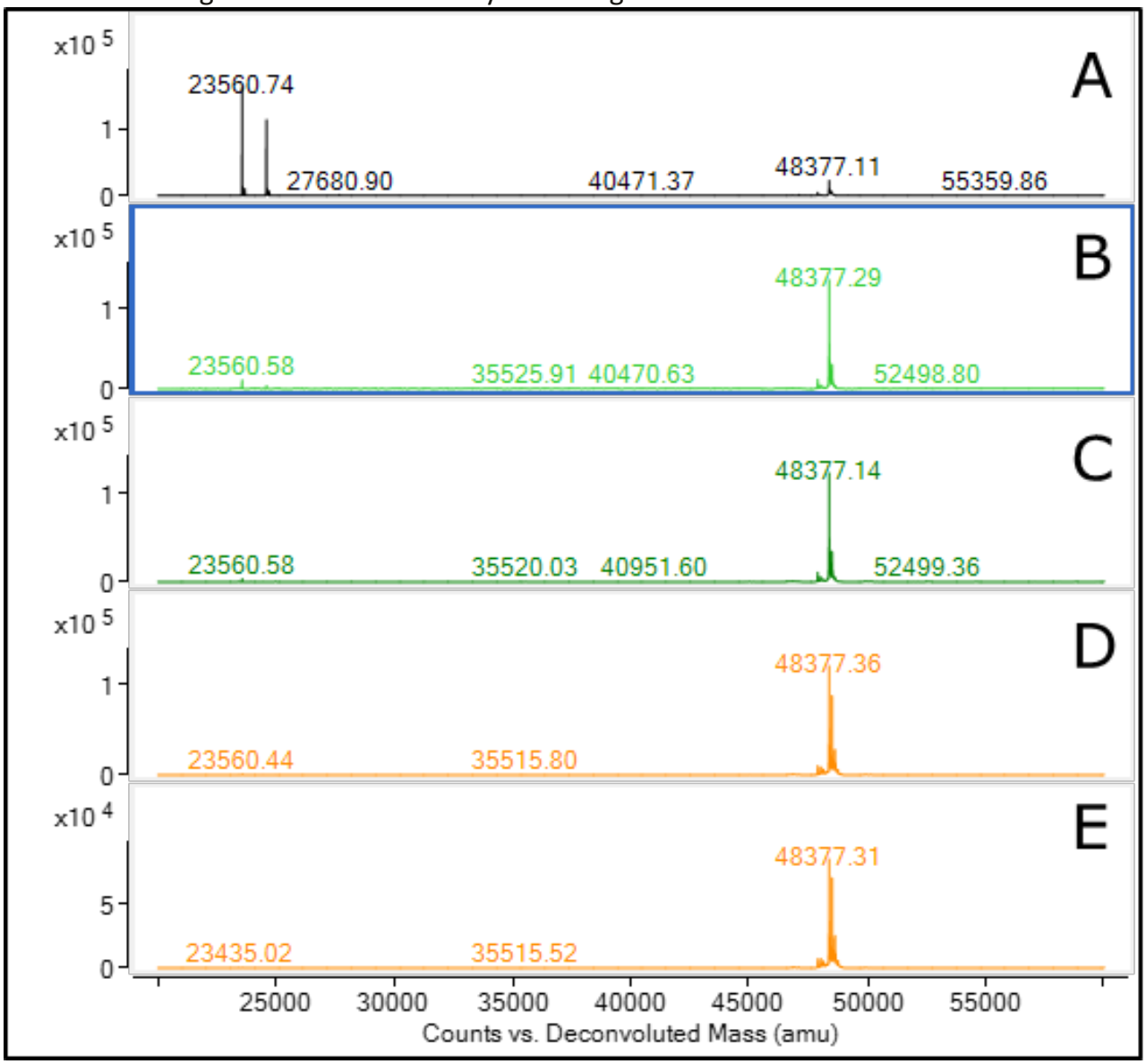


Figure S32. *The reaction between reduced Rem-Fab and reagent* ***1*** *quenched with NEM at different time points analysed by comparison of the deconvoluted masses of the reduced and oxidised Fab following LCMS. Reduced Rem-Fab (25 µM) was mixed with reagent* ***1*** *(75 µM). A sample was withdrawn and added to a vial containing 2000 eq. of NEM, which stopped the reaction at the following time points: A) directly after mixing - 0 minutes, B) 5 minutes, C) 10 minutes, D) 30 minutes, and E) 60 minutes. The data suggests that 10 minutes is sufficient to nearly quantitatively staple the protein.*

Rit-Fab experiments

Rit-Fab native

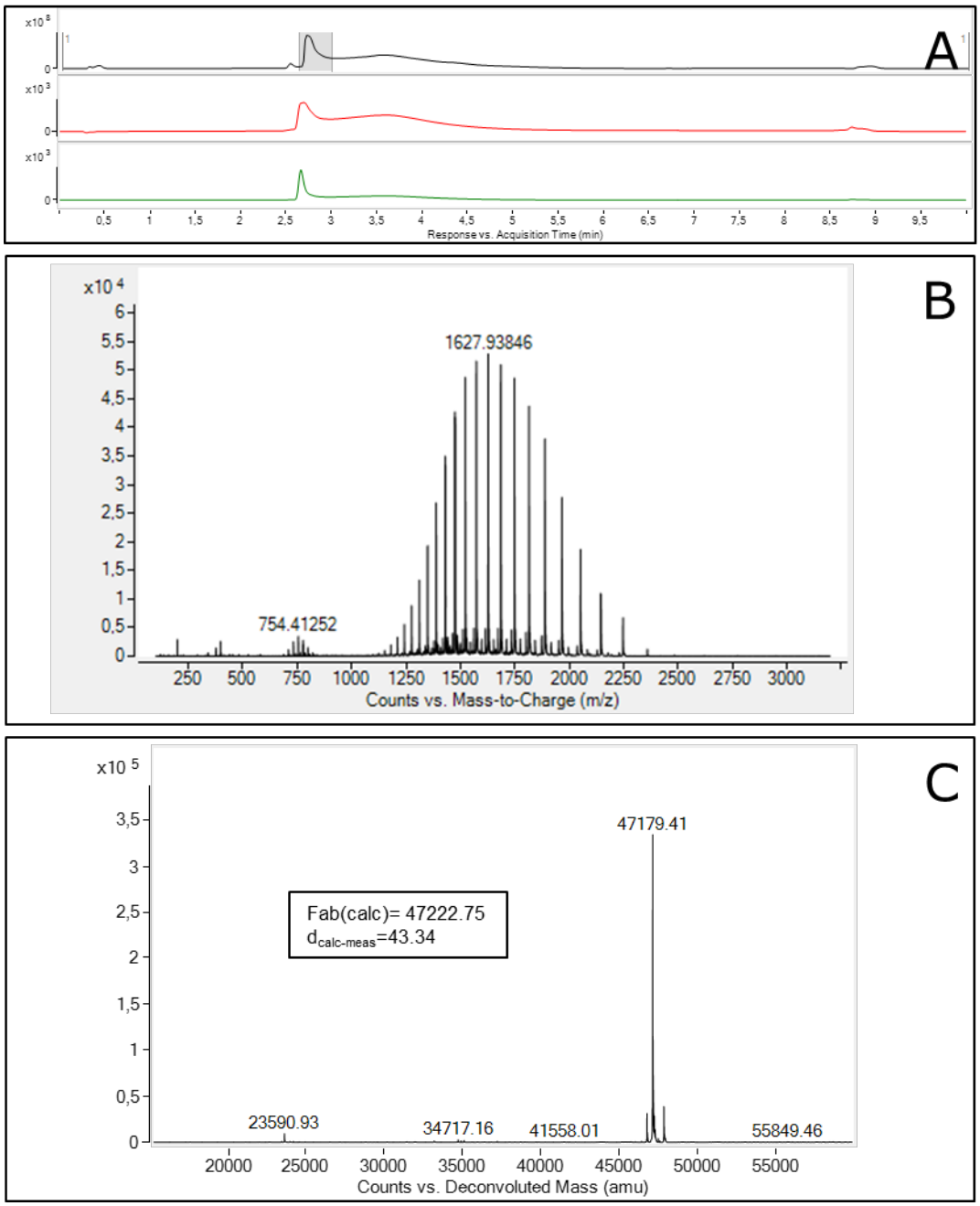


Figure S33. *The ÄKTA-purified Rit-Fab following papain digestion of Rituxan® analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B.*

Rit-Fab reduced

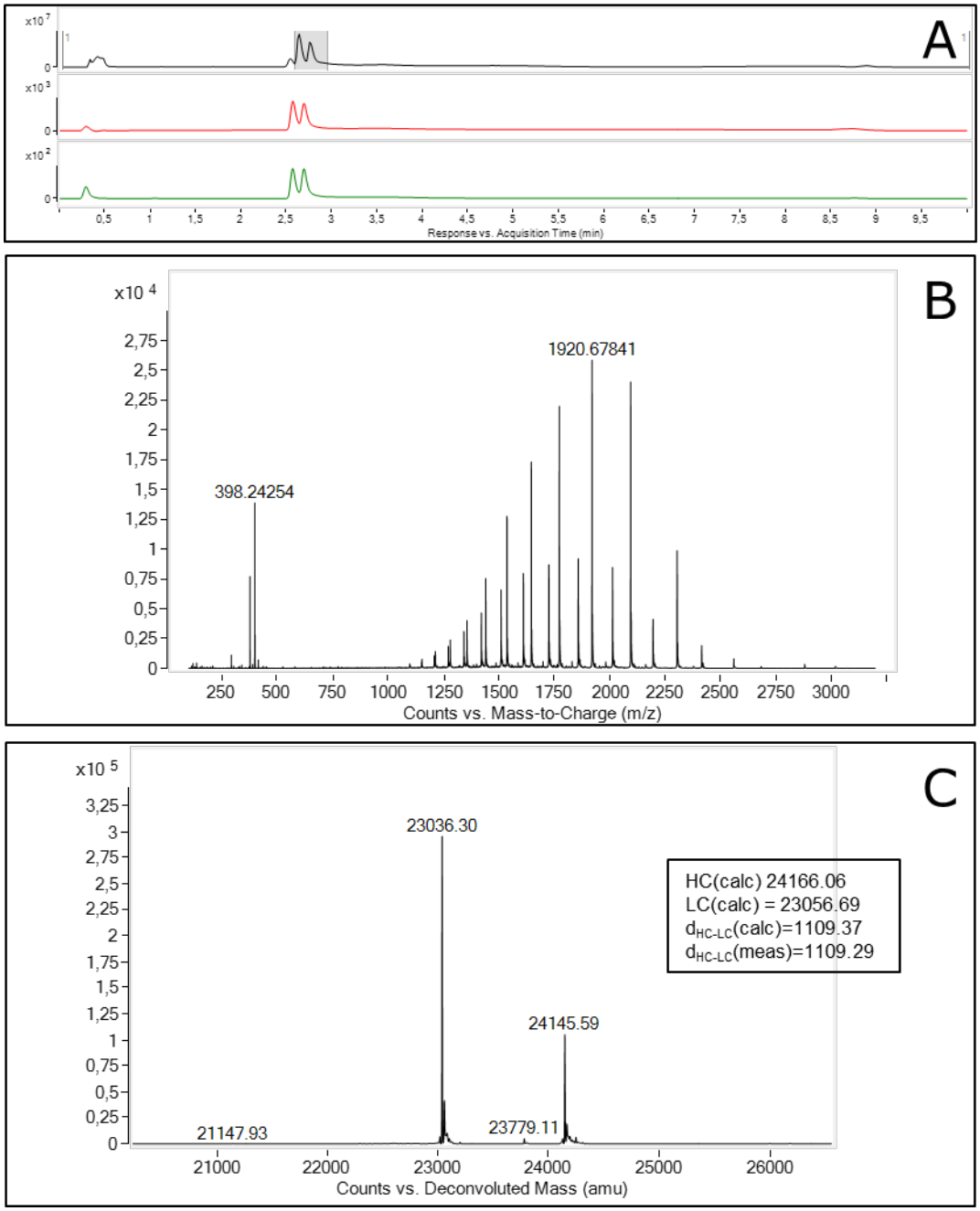


Figure S34. *The reduction of Rit-Fab with DTT analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses of light chain and heavy chain.*

Rit-Fab rebridged reaction with reagent **1**

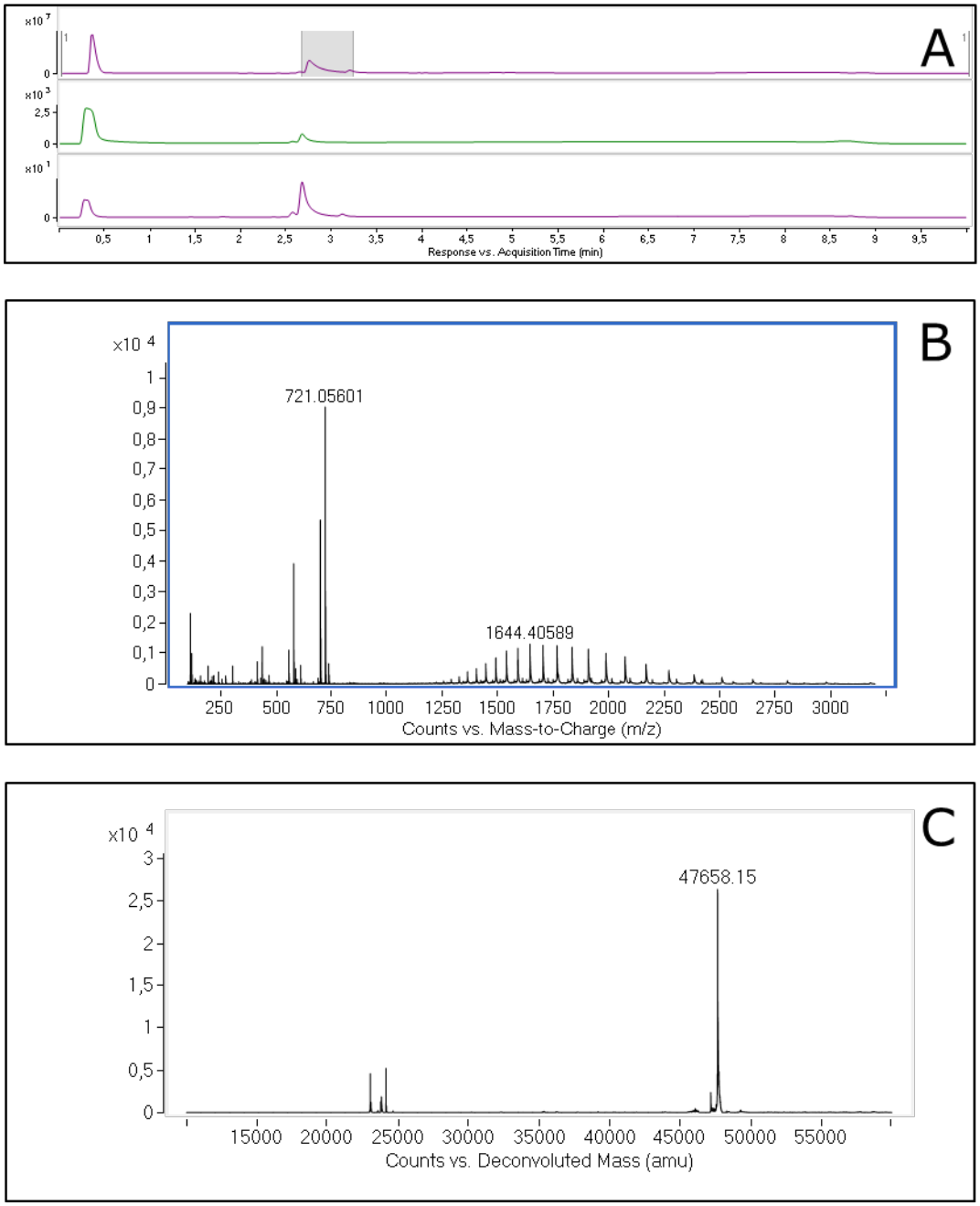


Figure S35. *The rebridged reaction between reduced Rit-Fab and reagent* ***1*** *analysed by LCMS.* ***A)*** *TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom)* ***B)*** *m/z of the scan range indicated by the grey box in A).* ***C)*** *Deconvolution of B. The mass of 47658.15 corresponds to the addition of 1 reagent molecule to the Fab. (Expected mass 47658.47 Da).*

Rit-Fab NEM conjugation reagent **1**

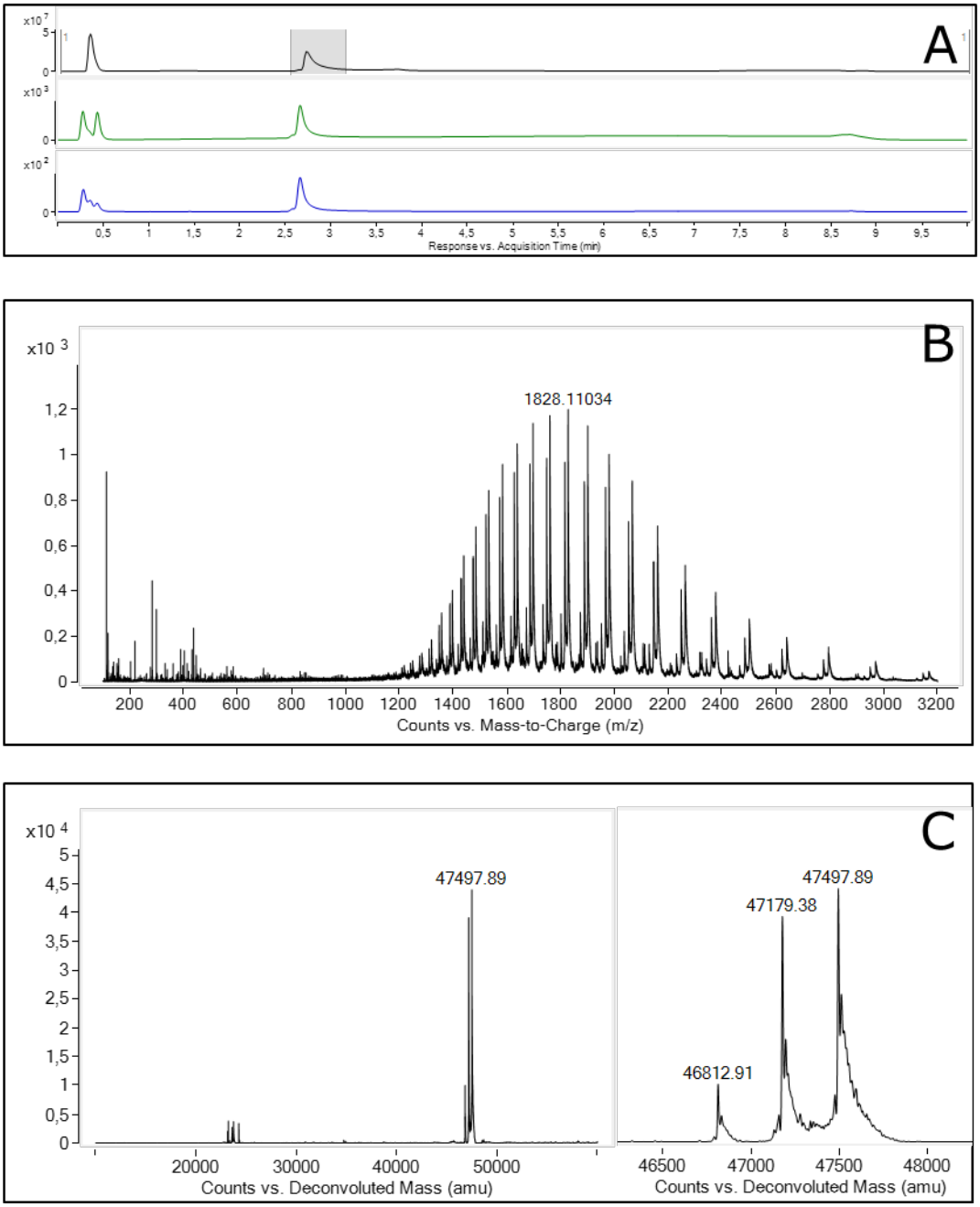


Figure S36. *The NEM-conjugation to pseudo-cysteine Rit-Fab analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvolution of B with masses. The mass of 47497.89 corresponds to the addition of 1 molecule of NEM to the handle. (Expected mass = 47498.52 Da)*

Rit-Fab rebridged reaction time study with reagent **1**

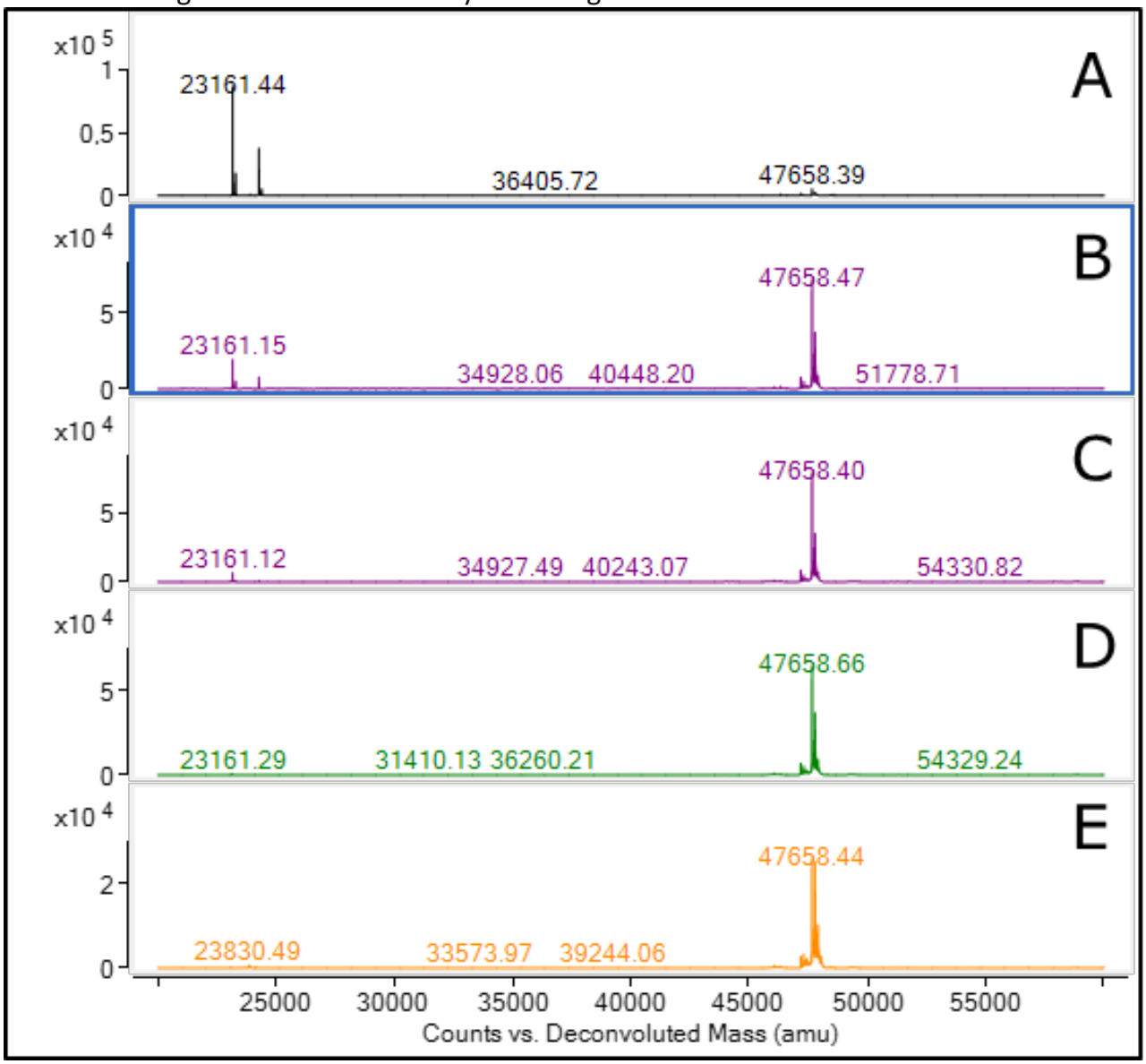


Figure S37. *The reaction between reduced Rit-Fab and reagent **1** quenched with NEM at different time points analysed by comparison to the deconvoluted masses of the reduced and oxidised Fab following LCMS. Reduced Rit-Fab (25 µM) was mixed with reagent **1** (75 µM). A sample was withdrawn and added to a vial containing 2000 eq. of NEM, which stopped the reaction at the following time points: A) directly after mixing - 0 minutes, B) 5 minutes, C) 10 minutes, D) 30 minutes, and E) 60 minutes. The data suggests that 15 minutes is sufficient to nearly quantitatively staple the protein.*

hGH experiments

Native hGH

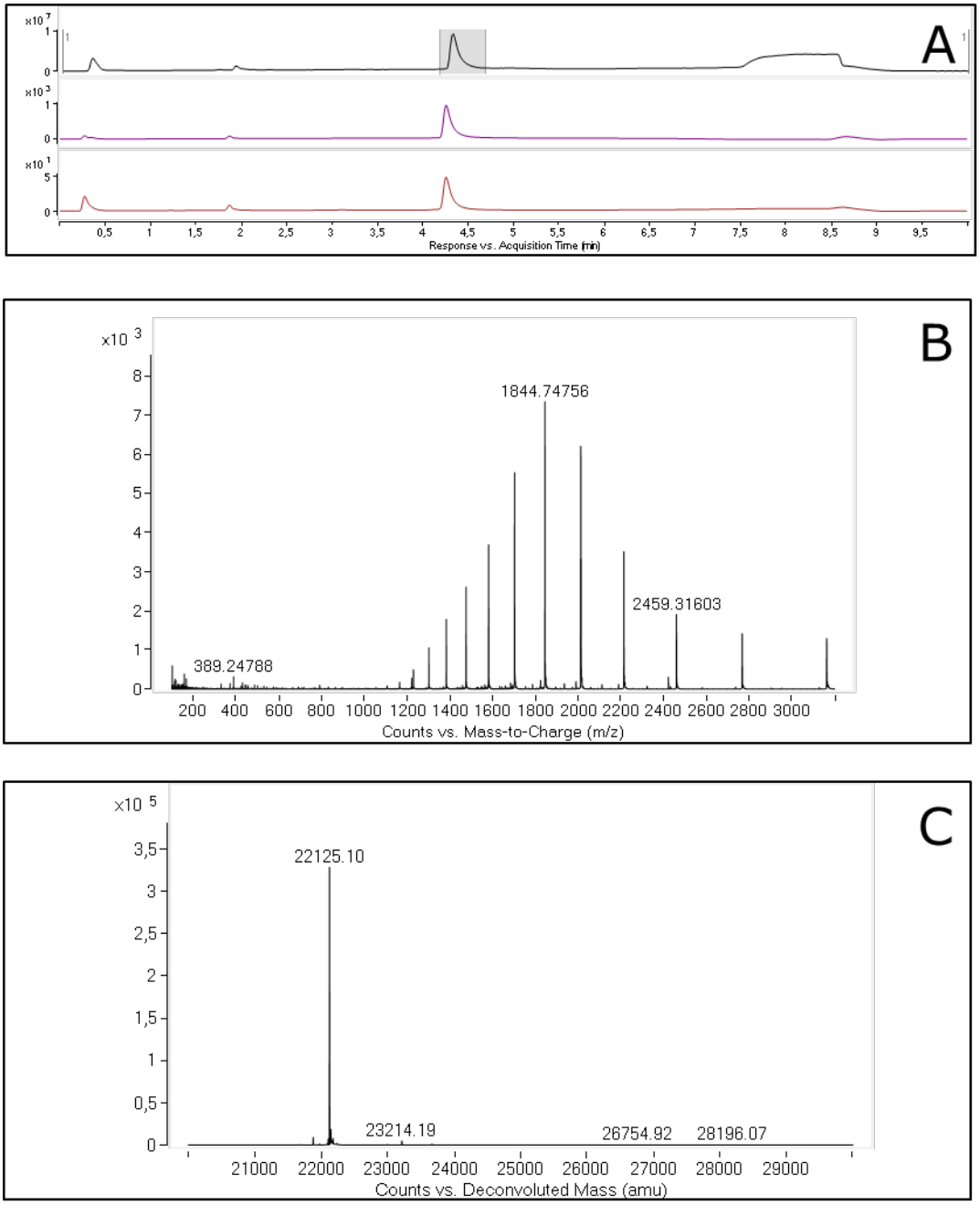


Figure S38. *Native hGH analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), DAD 280 nm (bottom), scan range (grey highlight) B) Counts vs. m/z of scan. C) deconvoluted masses.*

Reduced hGH

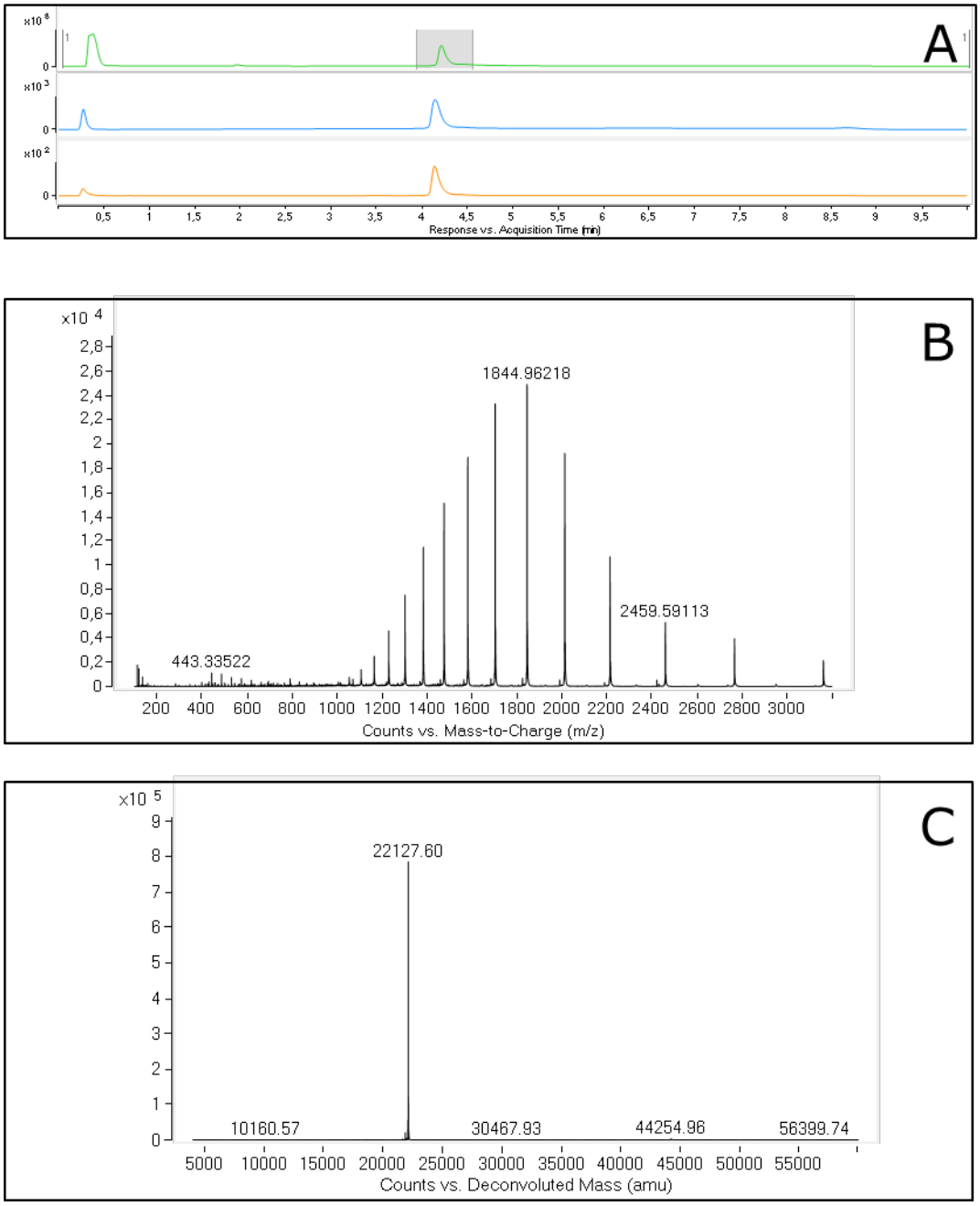


Figure S39. *The reduction of hGH with 2.5 eq. TCEP analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), DAD 280 nm (bottom), scan range (grey highlight) B) Counts vs. m/z of scan. C) deconvoluted masses. The mass of 22127.60 Da corresponds to +2H. (Expected mass 22127 Da)*

Reduced hGH NEM conjugated

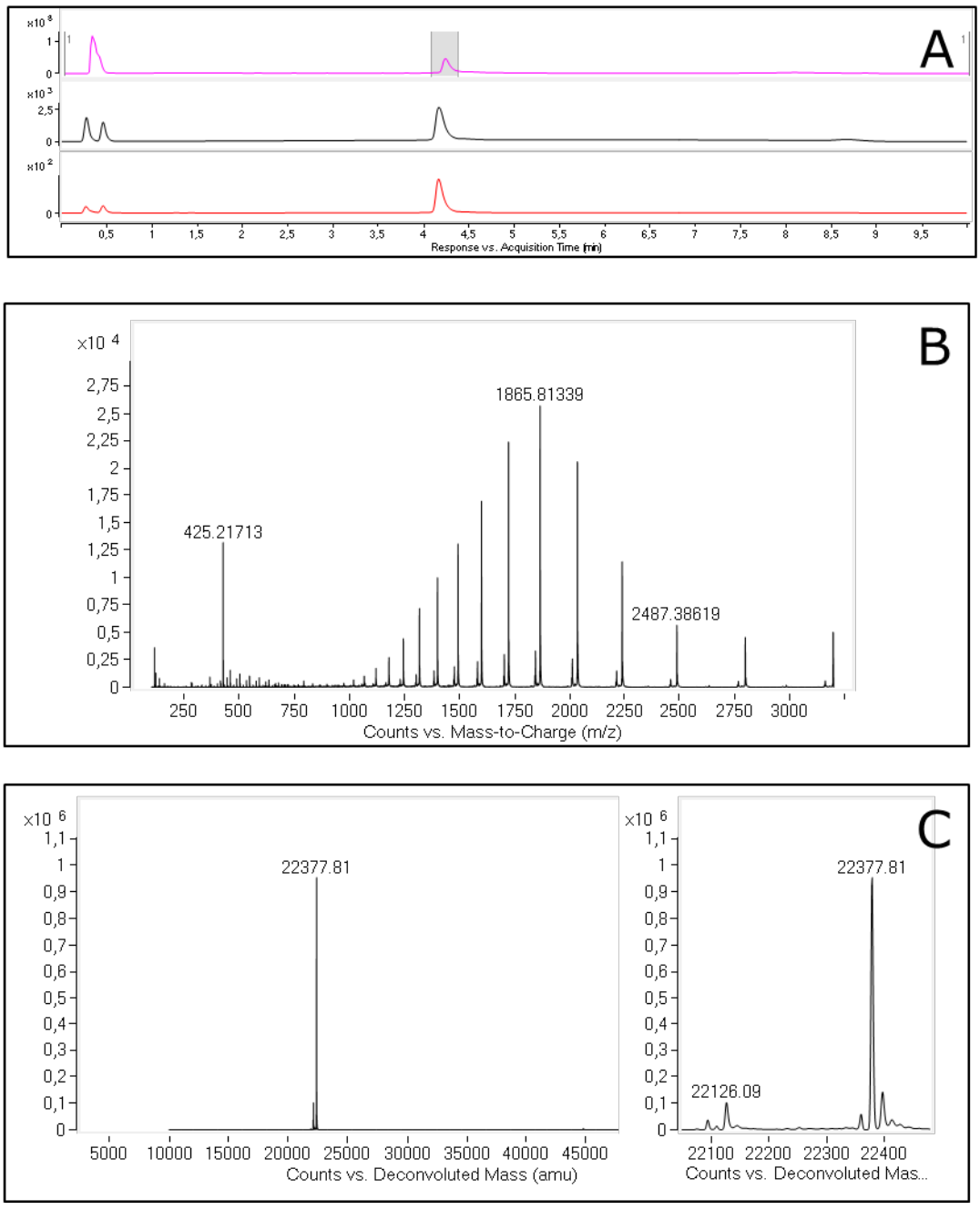


Figure S40. *Reaction of reduced hGH with NEM analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), DAD 280 nm (bottom), scan range (grey highlight) B) Counts vs. m/z of scan. C) deconvoluted masses. The mass of 22377.81 corresponds to the addition of 2 molecules of NEM (Expected mass = 22377.22 Da).*

hGH rebridged reaction with reagent **2**

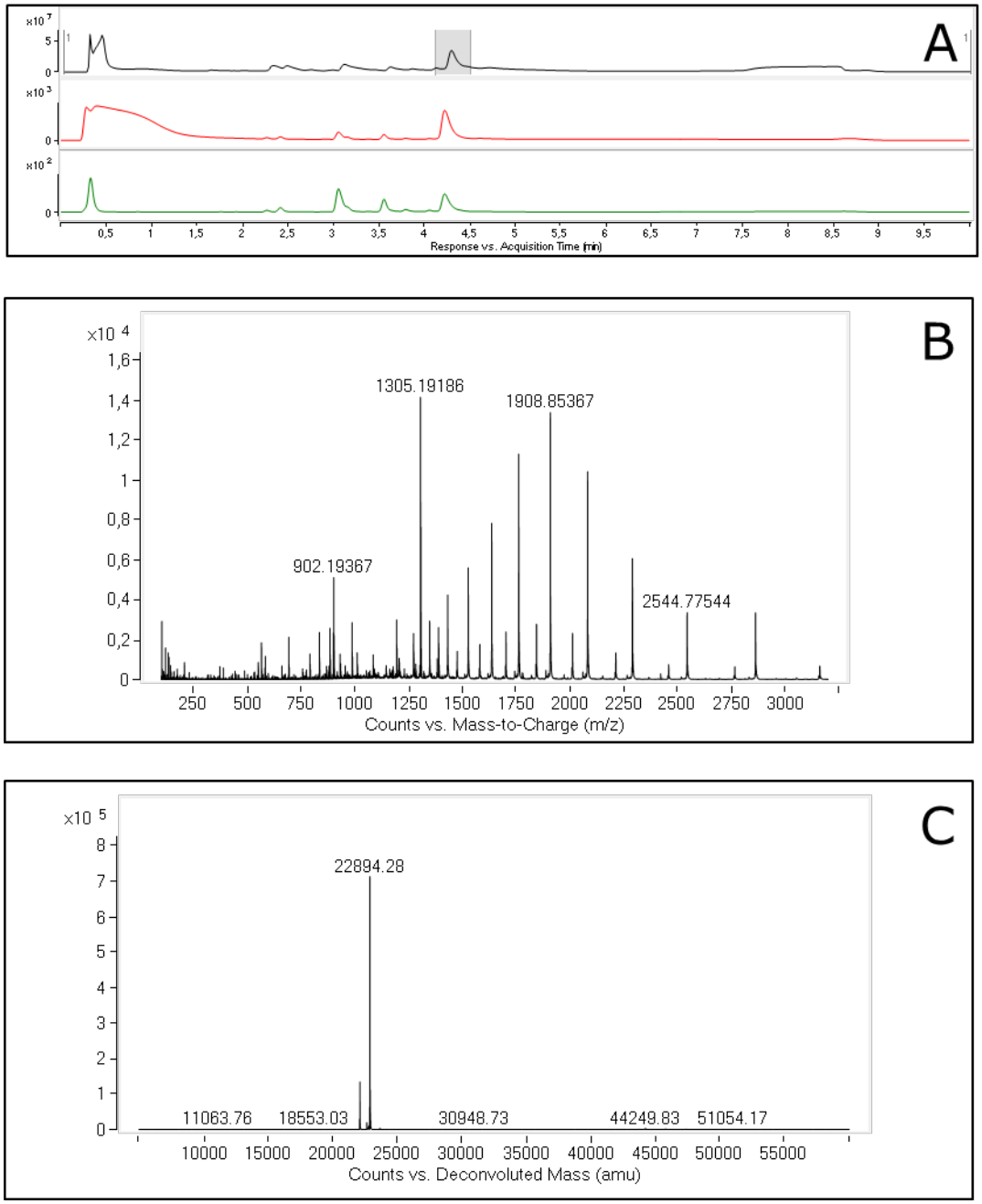


Figure S41. *The reaction of reduced hGH with reagent **2** analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvoluted masses. The mass of 22894.28 corresponds to the rebridging with a single reagent **2** molecule (Expected mass = 22894.31 Da).*

hGH reductive amination of reagent **2**

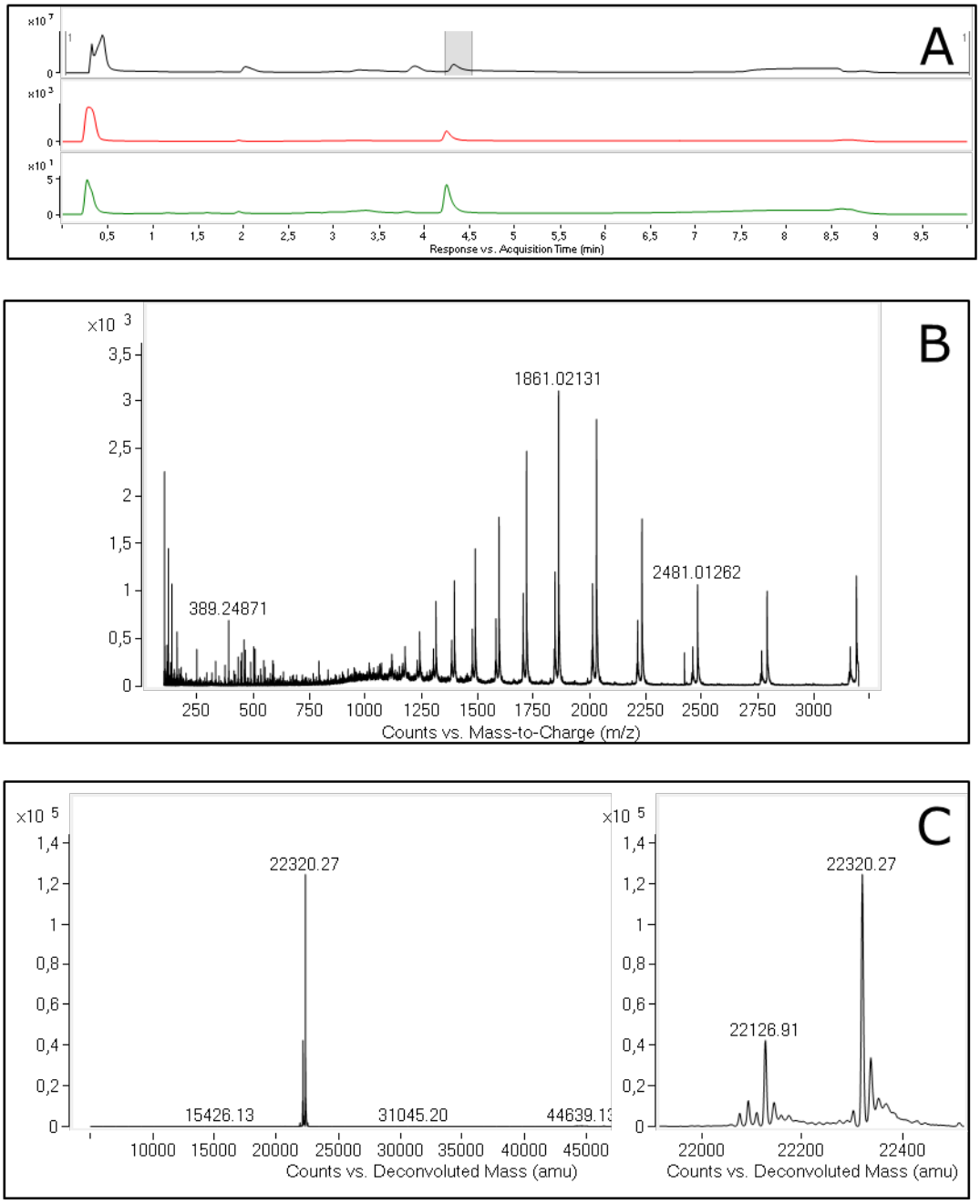


Figure S42. *The reduced reaction mixture of the reductive amination of reagent* ***2*** *rebridged hGH analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvoluted masses. The mass of 22320.27 corresponds to the addition of 1 pseudo-cysteine handle (Expected mass = 22320.16 Da).*

hGH reoxidation with dhAA followed by NEM conjugation

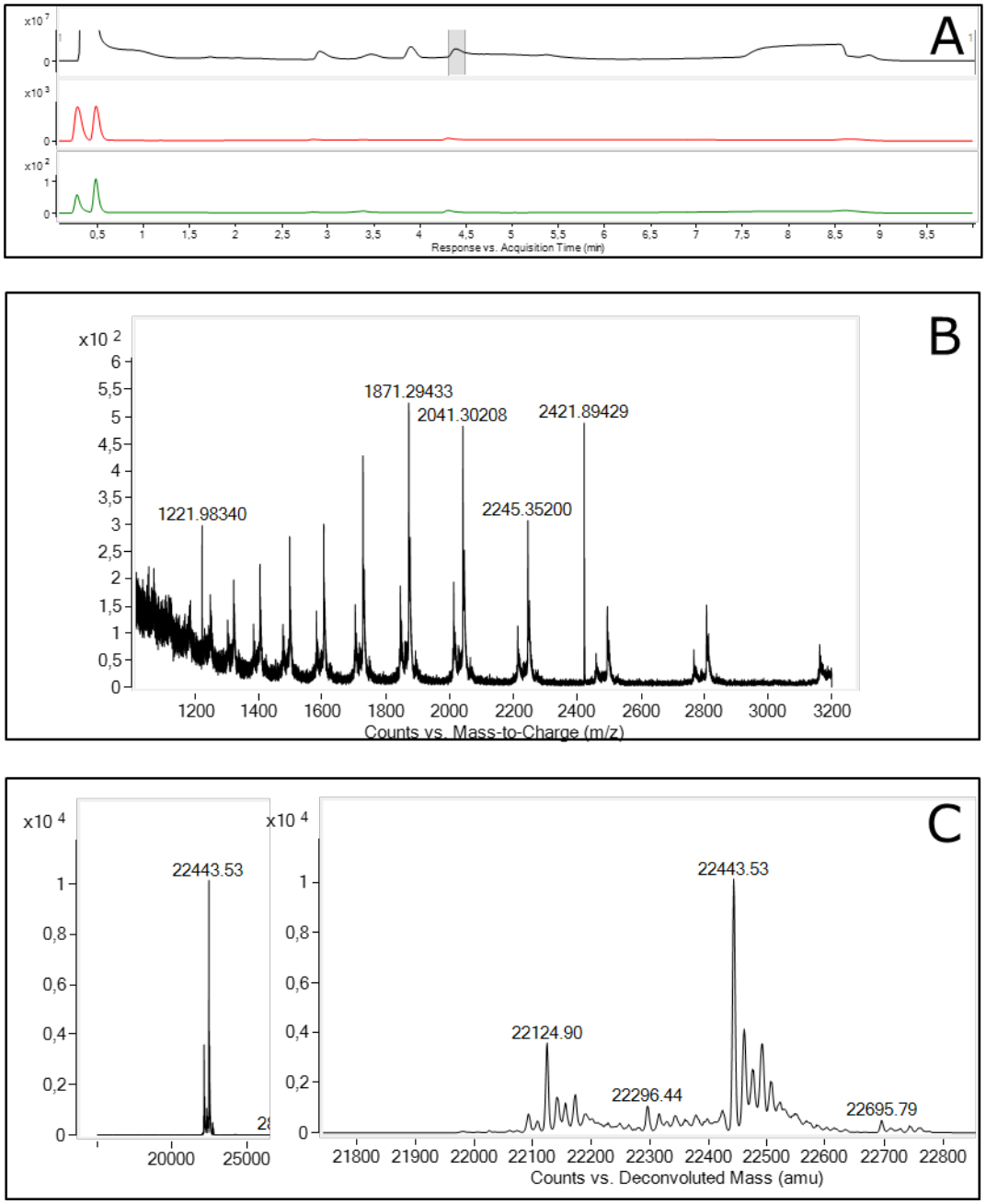


Figure S43. *The reduced reaction mixture following conjugation of NEM to the reagent* ***2*** *installed thiol handle on hGH analysed by LCMS. A) Chromatograms, TIC (top), DAD 214 nm (middle), and DAD 280 nm (bottom). The scan range is indicated by the grey box. B) Counts vs. m/z of scan. C) Deconvoluted masses. The mass of 22443.53 Da corresponds to the addition of 1 NEM to the pseudo-cysteine handle (Expected mass = 22444.21Da).*

SDS-PAGE data

Her-Fab PEGylation and DNA-conjugation

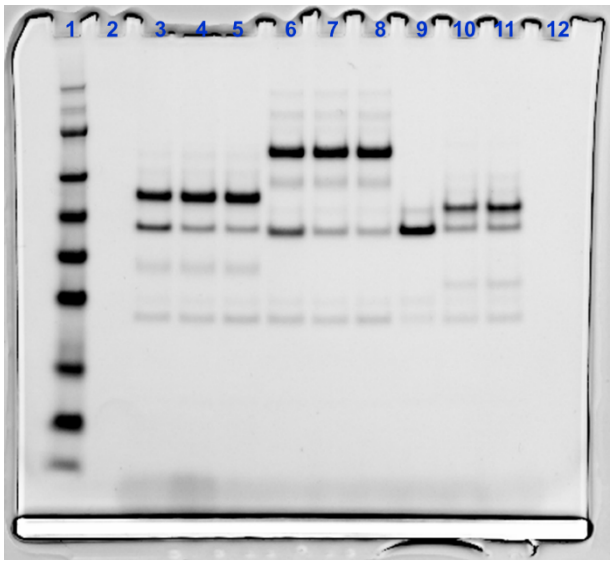


Figure S44. *The reaction mixture after conjugation to PEG-maleimide or DNA-maleimide following the installation of a pseudo-cys handle in Her-Fab by either reagent **1**, **2** or **3** analysed by 4-12% SDS-PAGE. Reactions were performed in PBS pH = 6.8 with 4 µM Her-Fab-pseudo-cysteine reaction mixture, in the presence of dhAA. PEG-maleimide (2 eq) or DNA-maleimide (1.2 eq) was added.*

Lanes: 1) Seeblue Plus 2 size marker; 3) Reaction mixture of 4 µM reagent 3 pseudo-cys with 5 kD; 4) Reaction mixture of reagent 2 pseudo-cys with 5 kDa PEG-maleimide; 5) Reaction mixture of reagent 1 pseudo-cys with 5 kDa PEG-maleimide; 6) Reaction mixture of reagent 3 pseudo-cys with 20 kDa PEG-maleimide; 7) Reaction mixture of reagent 2 pseudo-cys with 20 kDa PEG-maleimide; 8) Reaction mixture of reagent 1 pseudo-cys with 20 kDa PEG-maleimide; 9)her-Fab; 10) Reaction mixture of reagent 2 pseudo-cys with ODN1 maleimide; 11) Reaction mixture of reagent 1 pseudo-cys with ODN1 maleimide. Stained for protein with Safestain.

Time study of reductive amination

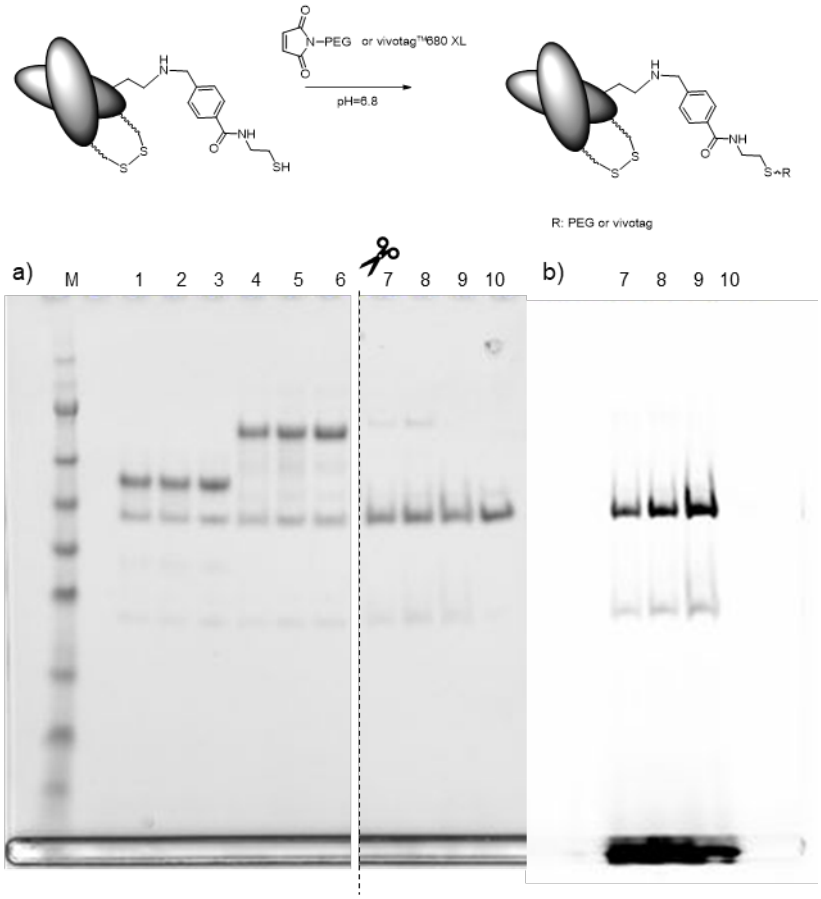


Figure S45. *Analysis of reductive amination conversion following 24, 48, or 72 hours of reaction time with reagent* ***2*** *analysed by 4-12% SDS-PAGE.*

(a) *The protein was rebridged with reagent* ***2*** *and reductive amination was performed. The reagent was released and filtered with desalting spin filters. The protein was reoxidised with dhAA (40 eq.) and reacted with 2 eq. 5 kDa PEG-maleimide or 20 kDa PEG-maleimide, or 20 eq. vivotag™680XL fluorophore maleimide.*

Lanes: 1) 24-hour reaction, 5 kDa PEG; 2) 48-hour reaction, 5 kDa PEG; 3) 72-hour reaction, 5 kDa PEG; 4) 24-hour reaction, 20 kDa PEG; 5) 48-hour reaction, 20 kDa PEG; 6) 72-hour reaction, 20 kDa PEG; 7) 24-hour reaction, vivotag; 8) 48-hour reaction, vivotag; 9) 72-hour reaction, vivotag; 10) Her-Fab. The gel was stained for protein with Safestain.

(b) *Image from (a) scanned for vivotag™680XL fluorescence. The bands all appear to give the same distribution between modified and unmodified protein.*

DNA conjugation of Ava-, Ben-, Erb-, and Her-Fab

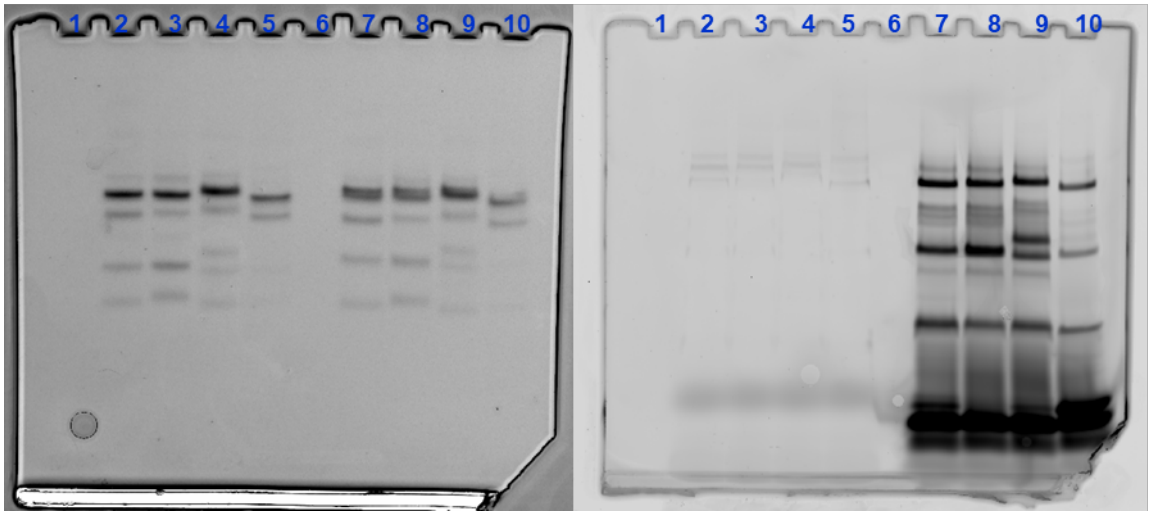


Figure S46. *The reaction mixture after conjugation to DNA-maleimide following the installation of a pseudo-Cys handle in Ava-, Ben-, Cet-, and Her-Fab analysed by 4-12% SDS-PAGE. Conditions: The Fab-pseudo-cysteine was reacted at 2-4 µM with DNA-maleimide (2 eq). The left image is scanned for protein stained by Safestain, the right image is scanned for Alexa488 fluorescence.*

Lanes ***2)*** *Ava-Fab DNA-maleimide reaction mixture;* ***3)*** *Ben-Fab DNA-maleimide reaction mixture;* ***4)*** *Erb-Fab DNA-maleimide reaction mixture;* ***5)*** *Her-Fab DNA-maleimide reaction mixture;* ***7)*** *Ava-Fab DNA-maleimide reaction mixture with 10 eq. Alexa488 modified complementary DNA;* ***8)*** *Ben-Fab DNA-maleimide reaction mixture with 10 eq. Alexa488 modified complementary DNA;* ***9)*** *Erb-Fab DNA-maleimide reaction mixture with 10 eq. Alexa488 modified complementary DNA;* ***10)*** *Her-Fab DNA-maleimide reaction mixture with 10 eq. Alexa488 modified complementary DNA.*

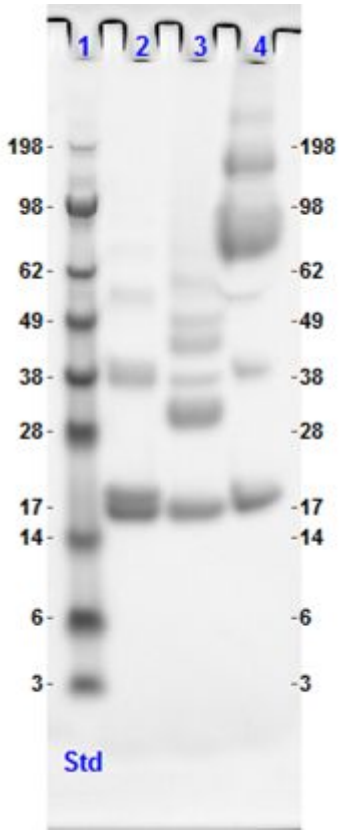
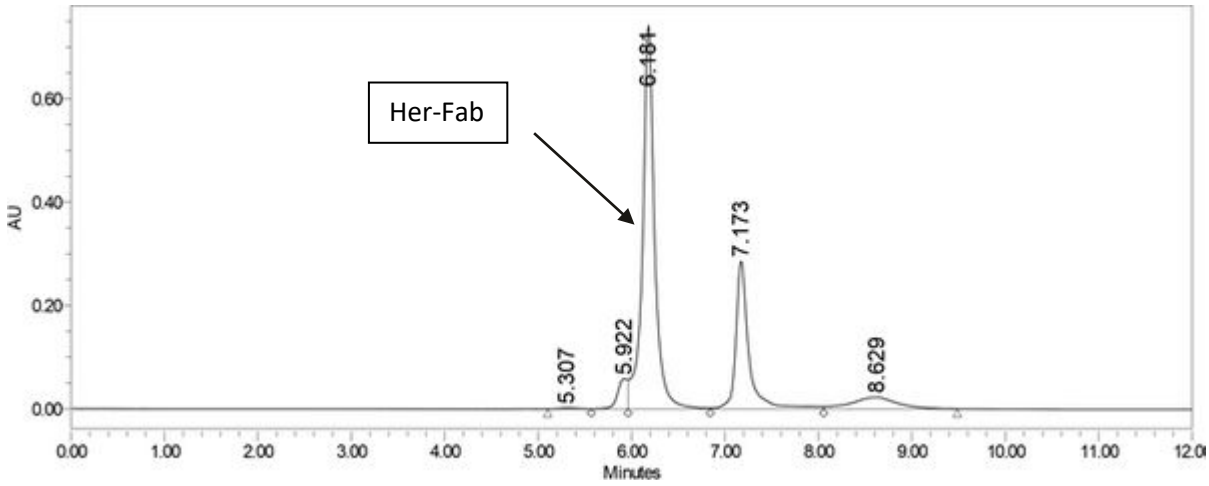


Figure S47. *PEGylation of hGH pseudo-cys handle following reaction with reagent* ***2*** *analysed by 4-12% SDS-PAGE. hGH (10 µM) was reacted with PEG-maleimide (3 eq) at pH=6.8 in PBS buffer.*

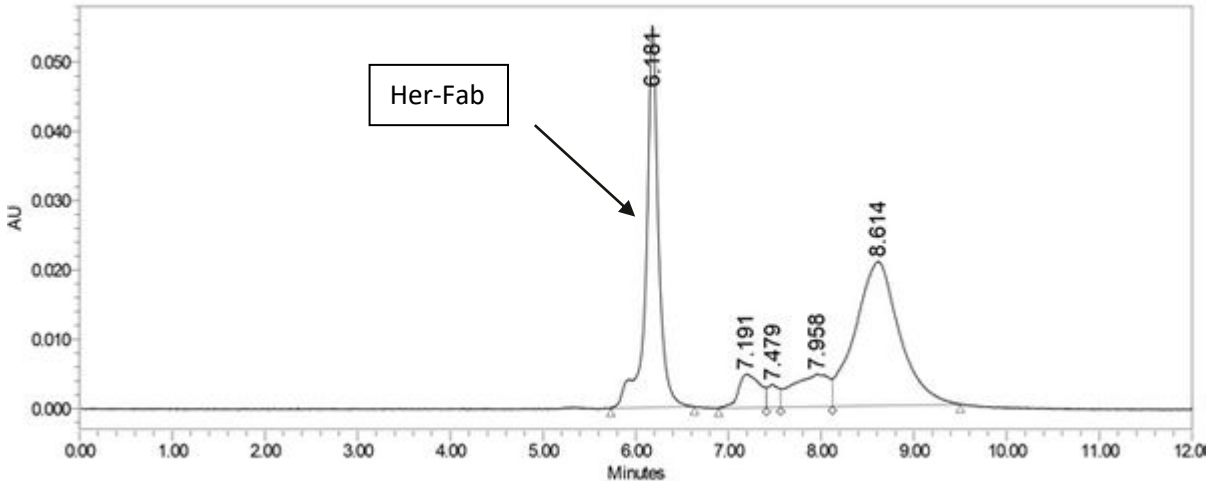
Lanes: ***1)*** *See Blue Plus 2 size marker;* ***2)*** *native hGH;* ***3)*** *hGH reaction product of reagent* ***3*** *conjugated with 5 kDa PEG;* ***4)*** *hGH reaction product of reagent* ***3*** *conjugated with 20 kDa PEG. Stained for protein with Safestain.*

UPLC data

SEC of native Her-Fab



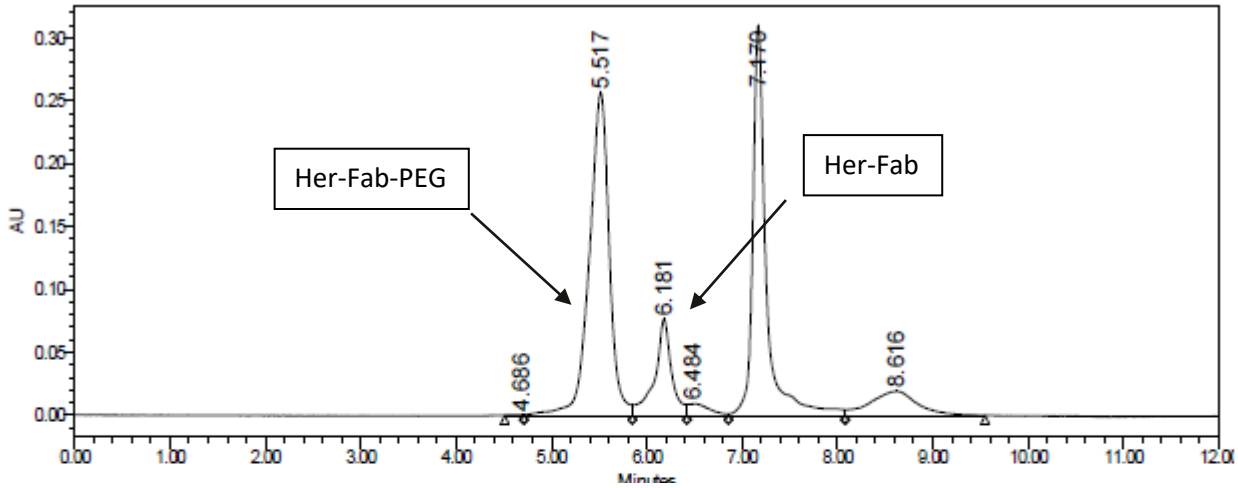
214 nm absorbance



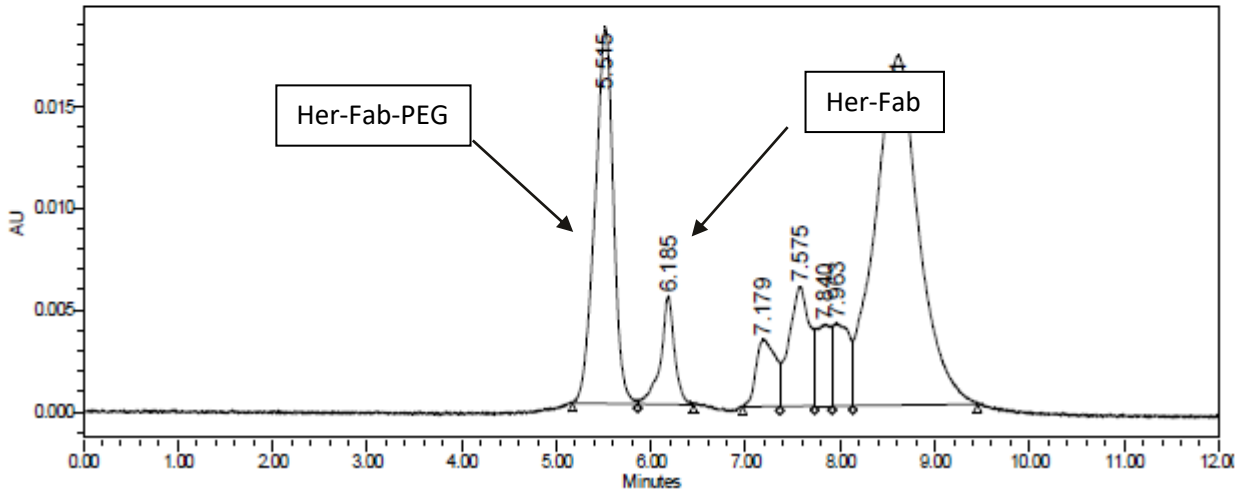
280 nm absorbance

Figure S48. *Native Her-Fab analysed by size exclusion chromatography (SEC). The peak at 6.18 min is the Fab.*

SEC of 5 kDa PEGylated Her-Fab made by reagent **1**



214 nm absorbance



280 nm absorbance

Figure S49. *The reaction of 5 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***1*** *analysed by SEC. Top: 214 nm abs. Bottom: 280 nm abs. The peak at 5.15 min corresponds to the PEGylated conjugate.*

SEC of 20 kDa PEGylated Her-Fab made by reagent **1**

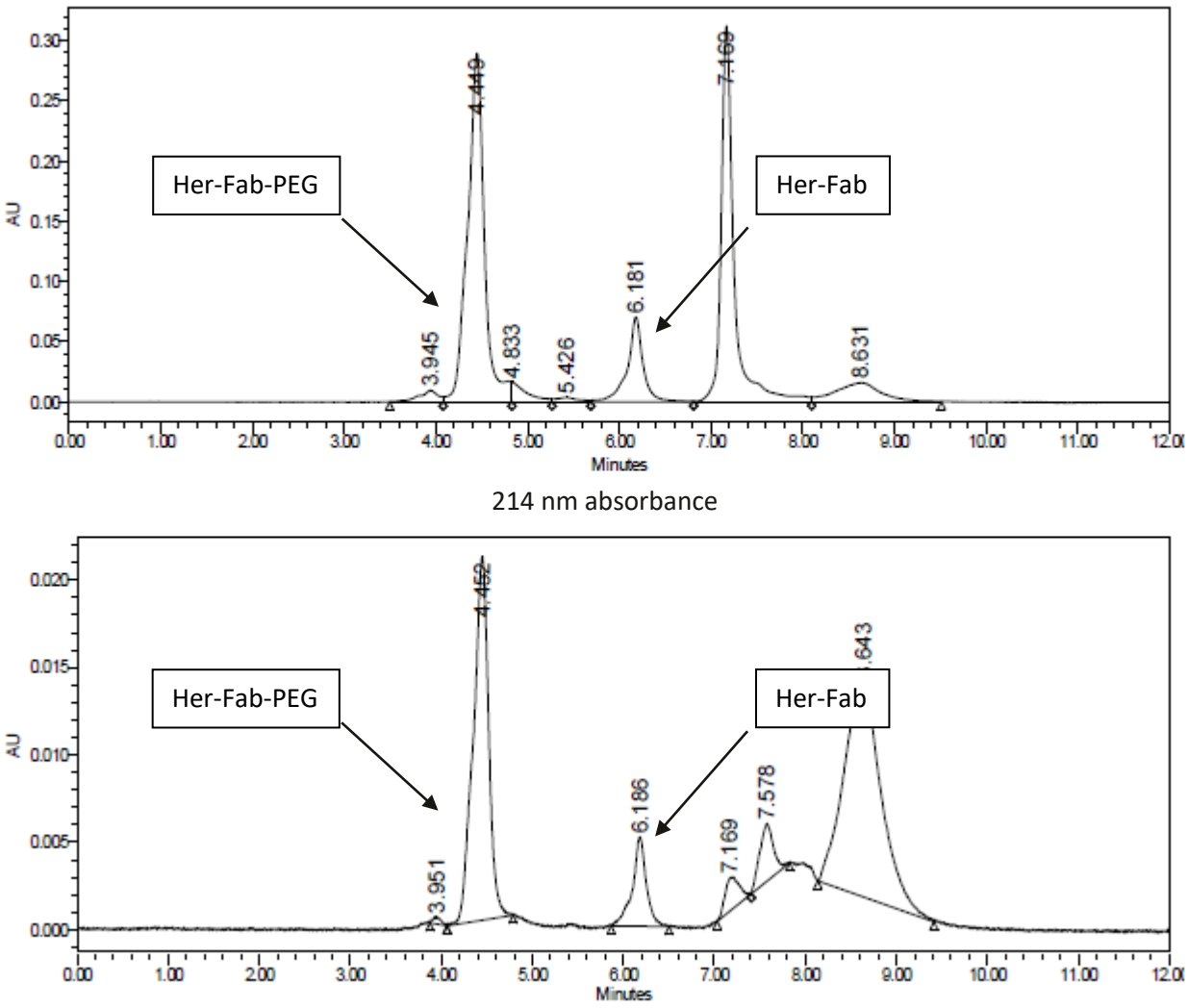
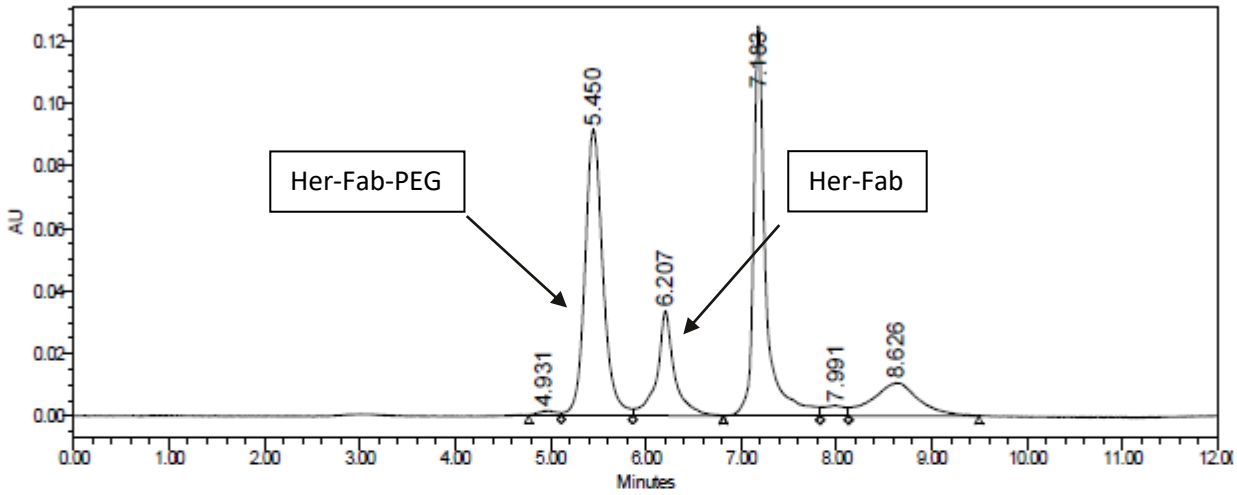
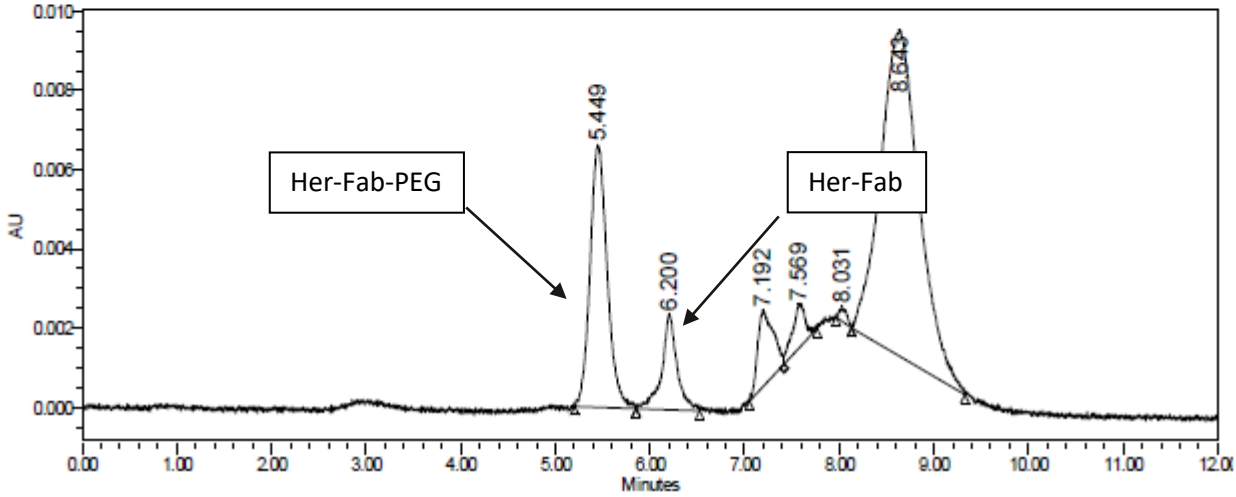


Figure S50. *The reaction of 20 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***1*** *analysed by SEC. Top: 214 nm abs. Bottom: 280 nm abs. The peak at 4.45 min corresponds to the PEGylated conjugate.*

SEC of 5 kDa PEGylated Her-Fab made by reagent **2**



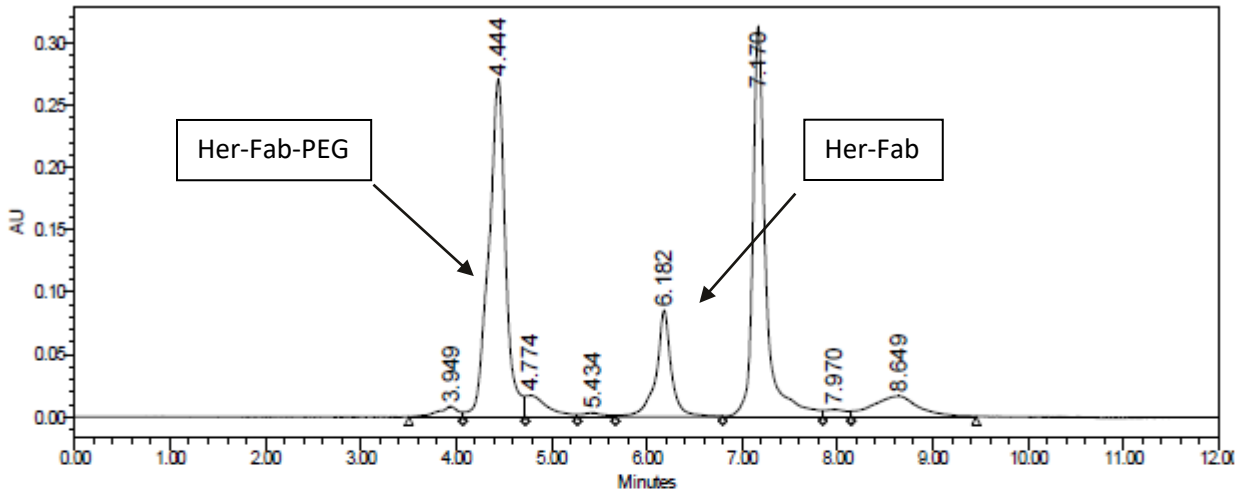
214 nm absorbance



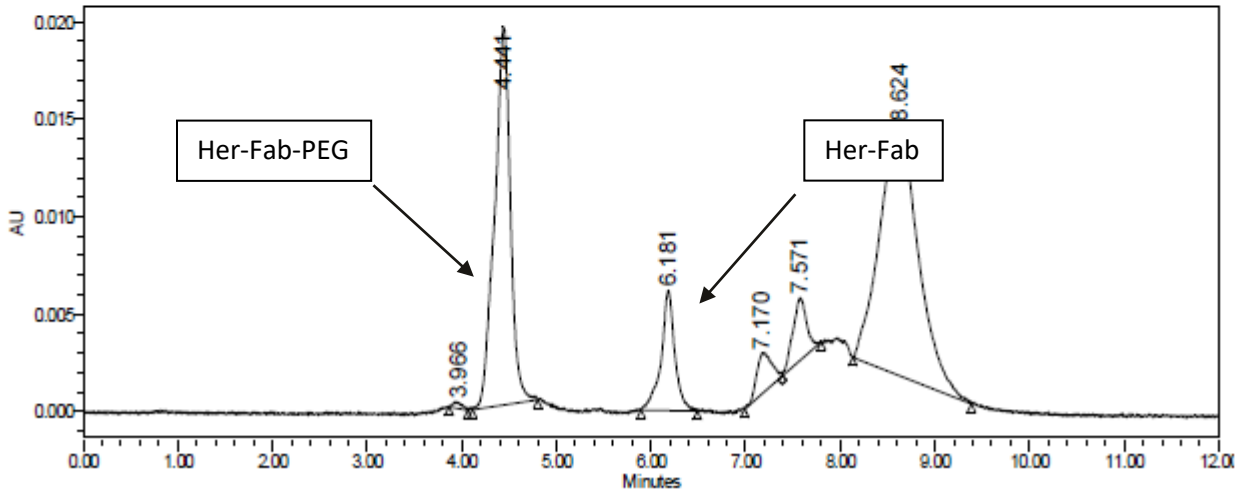
280 nm absorbance

Figure S51. *The reaction of 5 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***2*** *analysed by SEC. Top: 214 nm abs. Bottom: 280 nm abs. The peak at 5.45 min corresponds to the pPEGylated conjugate.*

SEC of 20 kDa PEGylated Her-Fab made by reagent **2**



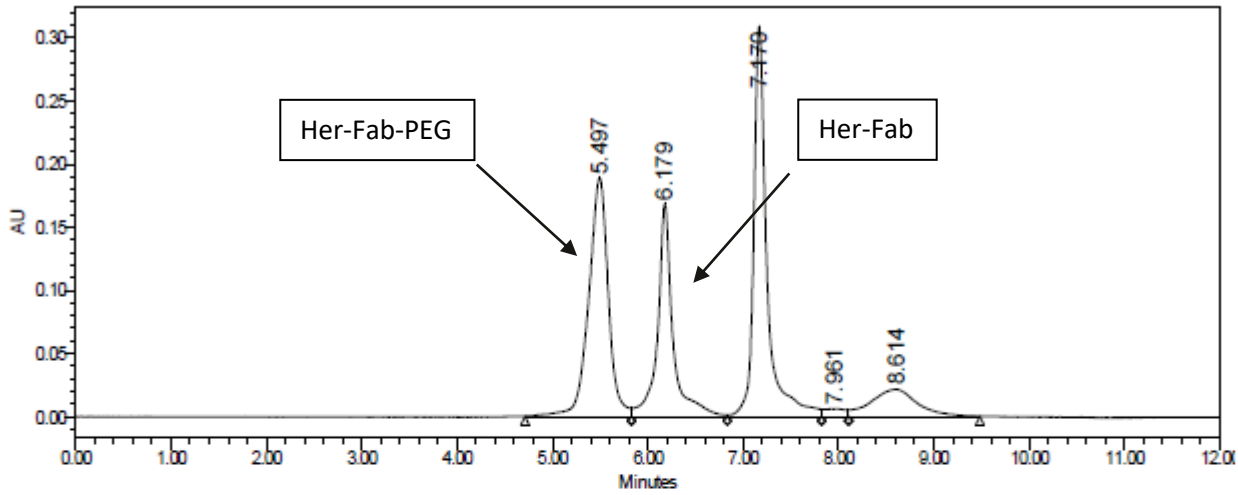
214 nm absorbance



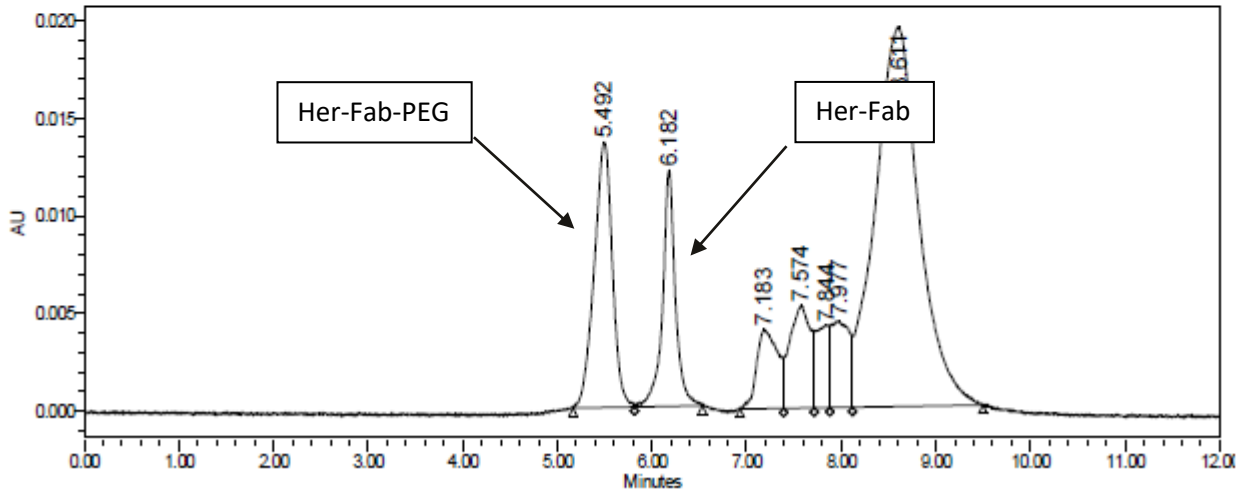
280 nm absorbance

Figure S52. *The reaction of 20 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***2*** *analysed by SEC. Top: 214 nm abs. Bottom: 280 nm abs. The peak at 4.44 min corresponds to the PEGylated conjugate.*

SEC of 5 kDa PEGylated Her-Fab made by reagent **3**



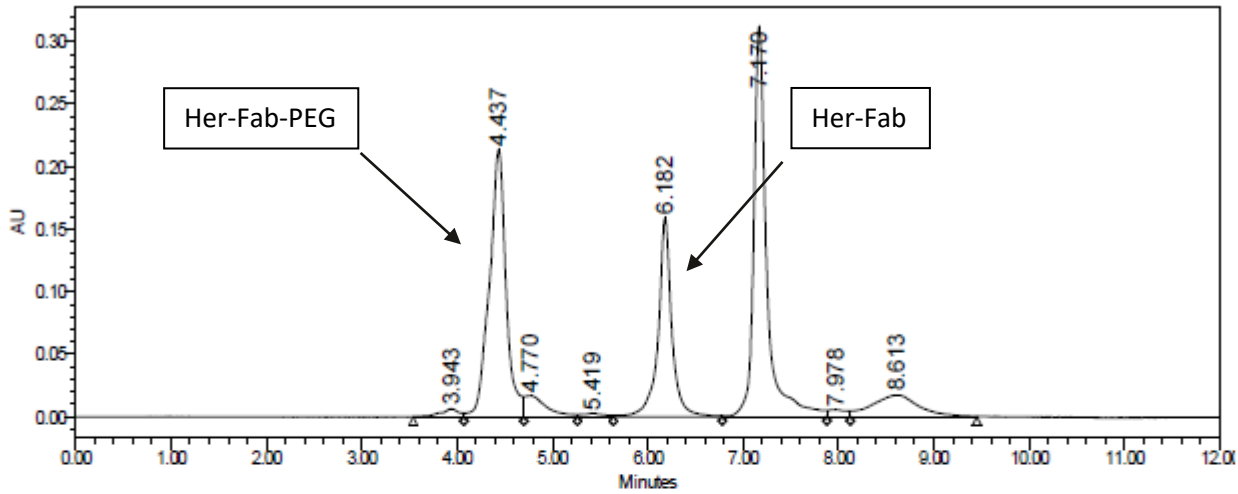
214 nm absorbance



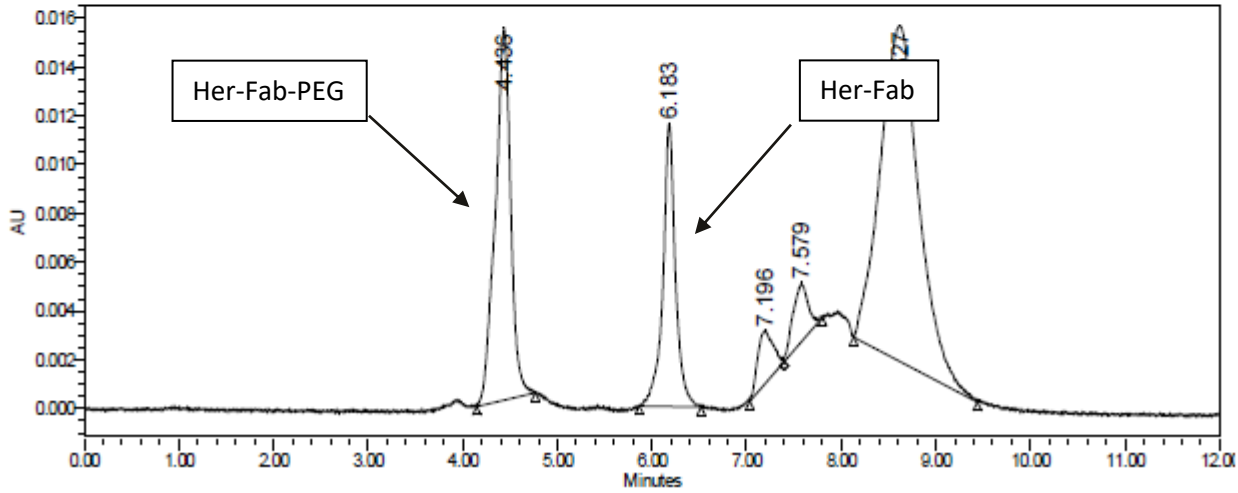
280 nm absorbance

Figure S53. *The reaction of 5 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***3*** *analysed by SEC. Top: 214 nm abs. Bottom: 280 nm abs. The peak at 5.49 min corresponds to the PEGylated conjugate.*

SEC of 20 kDa PEGylated Her-Fab made by reagent **3**



214 nm absorbance



280 nm absorbance

Figure S54 .*The reaction of 20 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***3*** *analysed by SEC****.*** *Top: 214 nm abs. Bottom: 280 nm abs. The peak at 4.44 min corresponds to the PEGylated conjugate.*

C4 RP-UPLC of reduced Her-Fab

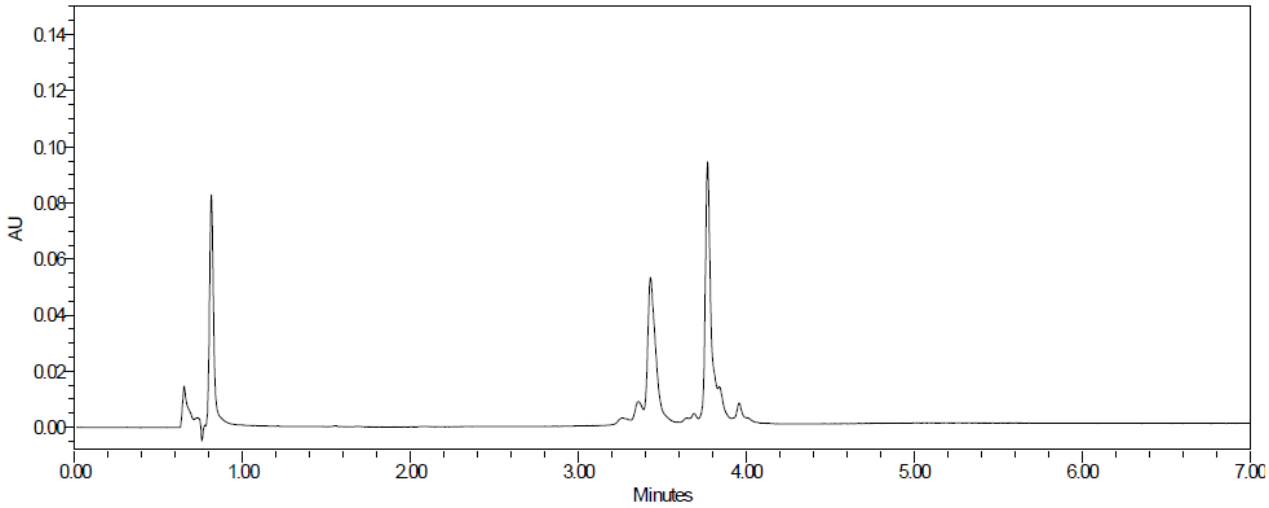
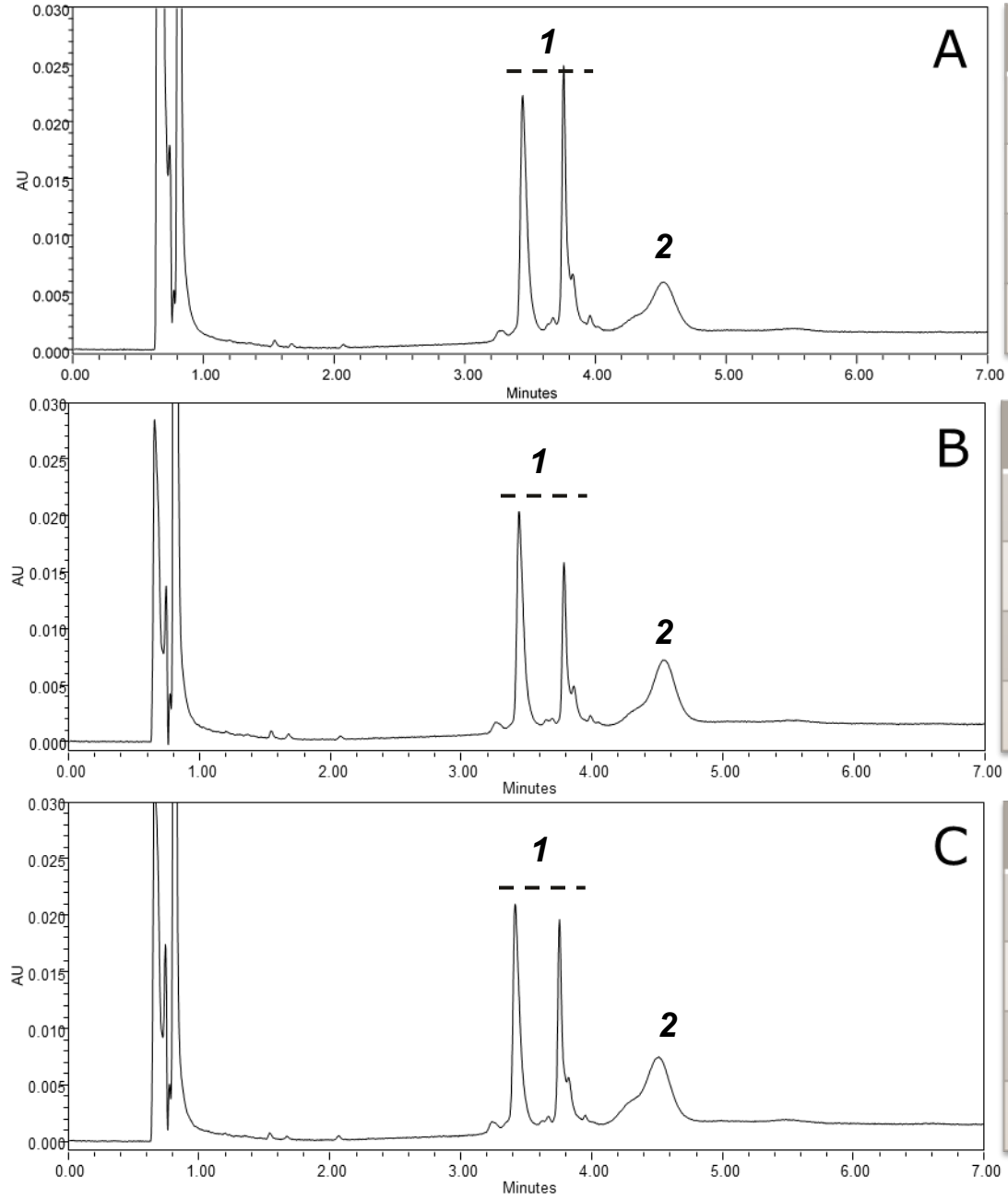


Figure S55. *Reduced Her-Fab analysed after DTT reduction by C4 RP-HPLC recording the absorbance at 280 nm.*

C4 RP-UPLC of reduced Her-Fab after 5 kDa PEGylation



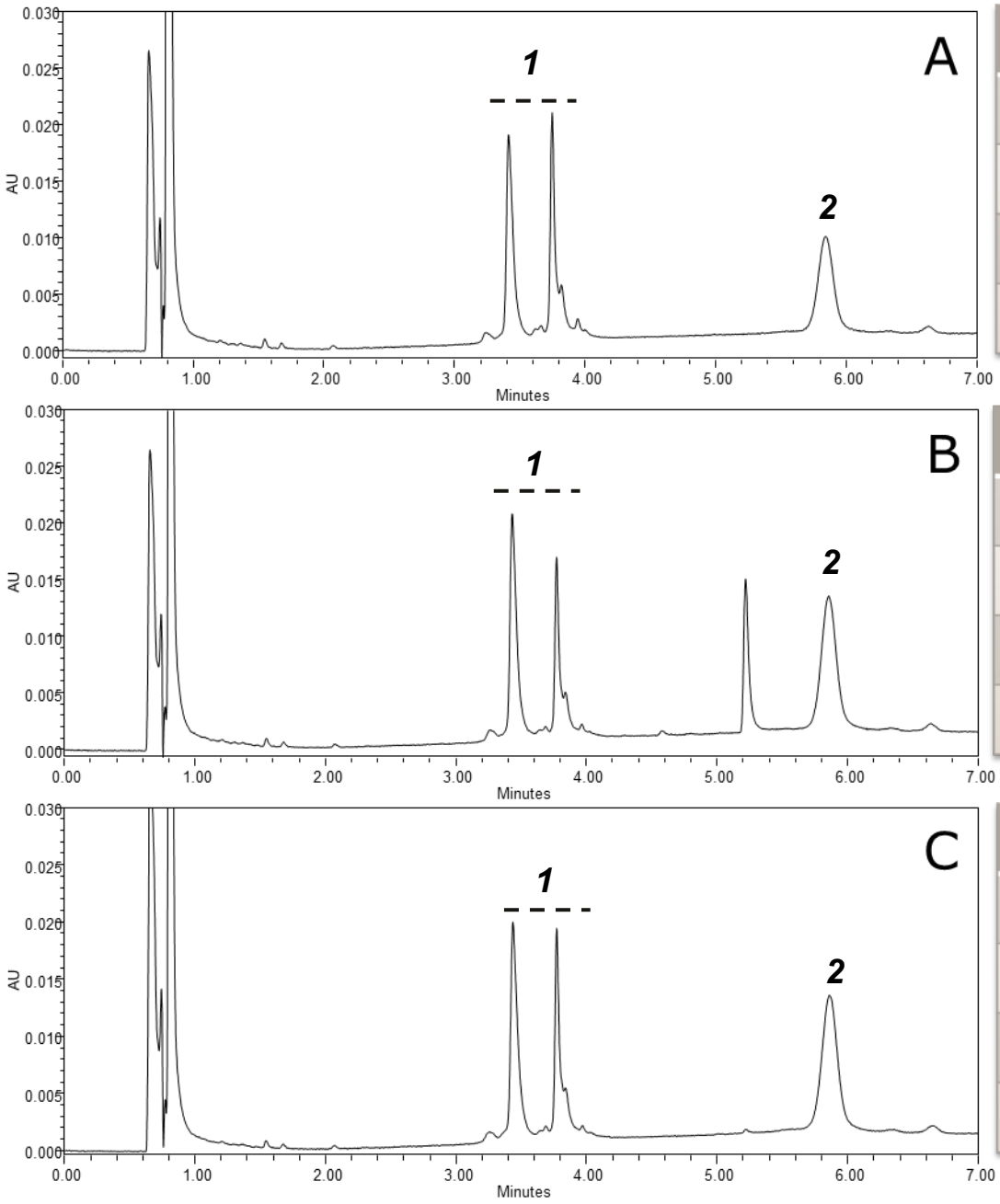
RT (min)	Area	% Area
3.443	74986	37.92
3.757	55000	27.82
3.826	11369	5.75
4.523	56372	28.51

RT (min)	Area	% Area
3.439	67553	36.04
3.783	35130	18.74
3.857	9913	5.29
4.540	74842	39.93

RT (min)	Area	% Area
3.417	70868	33.43
3.756	42724	20.16
3.826	10395	4.90
4.517	87974	41.50

Figure S56. *The reaction of 5 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent **1, 2** or **3** as analysed after DTT reduction by C4 RP-HPLC recording the absorbance at 280 nm. 1 denotes LC and HC fragments, 2 denotes LC or HC modified with PEG. A) Reagent **1** reaction. B) Reagent **2** reaction. C) Reagent **3** reaction.*

C4 RP-HPLC of reduced Her-Fab after 20 kDa PEGylation



RT (min)	Area	% Area
3.413	64246	33.20
3.746	47672	24.64
3.817	13854	7.16
5.842	67720	35.00

RT (min)	Area	% Area
3.430	65789	32.41
3.770	35683	17.58
3.838	8825	4.35
5.856	92681	45.66

RT (min)	Area	% Area
3.437	67192	30.97
3.774	42081	19.39
3.841	11594	5.34
5.860	96109	44.29

Figure S57. *The reaction of 20 kDa PEG-maleimide with pseudo-cys handle containing Her-Fab made through reagent* ***1, 2*** *or* ***3*** *as analysed after DTT reduction by C4 RP-HPLC recording the absorbance at 280 nm. 1 denotes LC and HC fragments, 2 denotes LC or HC modified with PEG. A) Reagent 1 reaction. B) Reagent 2 reaction. C) Reagent 3 reaction. The 5.2 min peak in B is an artefact.*

Flow Cytometry

Protocol

For flow cytometry, 200.000 cells/well (A431 (EGFR+) or SKBR3 (Her2+)) were seeded in a 24-well plate and allowed to attach overnight. Prior to the analysis, the SEC-purified Fab-DNA conjugate was annealed to an ATTO488 conjugated complementary ODN at 1 eq. for 30 min. The cells were then treated with 100 nM conjugate-fluorophore (ATTO488 or vivotag-680) in fresh cell media with 10% PBS for 1 h at 37 ºC. Blank samples were treated with fresh cell media with 10 % PBS. For the competition experiments, cells were charged with 1 µM free antibody (Cetuximab or Trastuzumab) for 10 min in fresh cell media with 10 % PBS before incubation with conjugate. After treatment, cells were washed three times with PBS and detached from the well using Trypsin-EDTA (0.05 %). The cells were then pelleted and Trypsin solution was removed. The cells were washed once with PBS and then suspended in PBS for analysis on a NovoCyte flow cytometer. The cells were gated for the main population and singlets. Chromatograms were smoothened using built-in software. A431 cells were grown in DMEM (10 % FBS) media and SKBR3 were grown in McCoy's 5a Medium modified (10 % FBS) media.

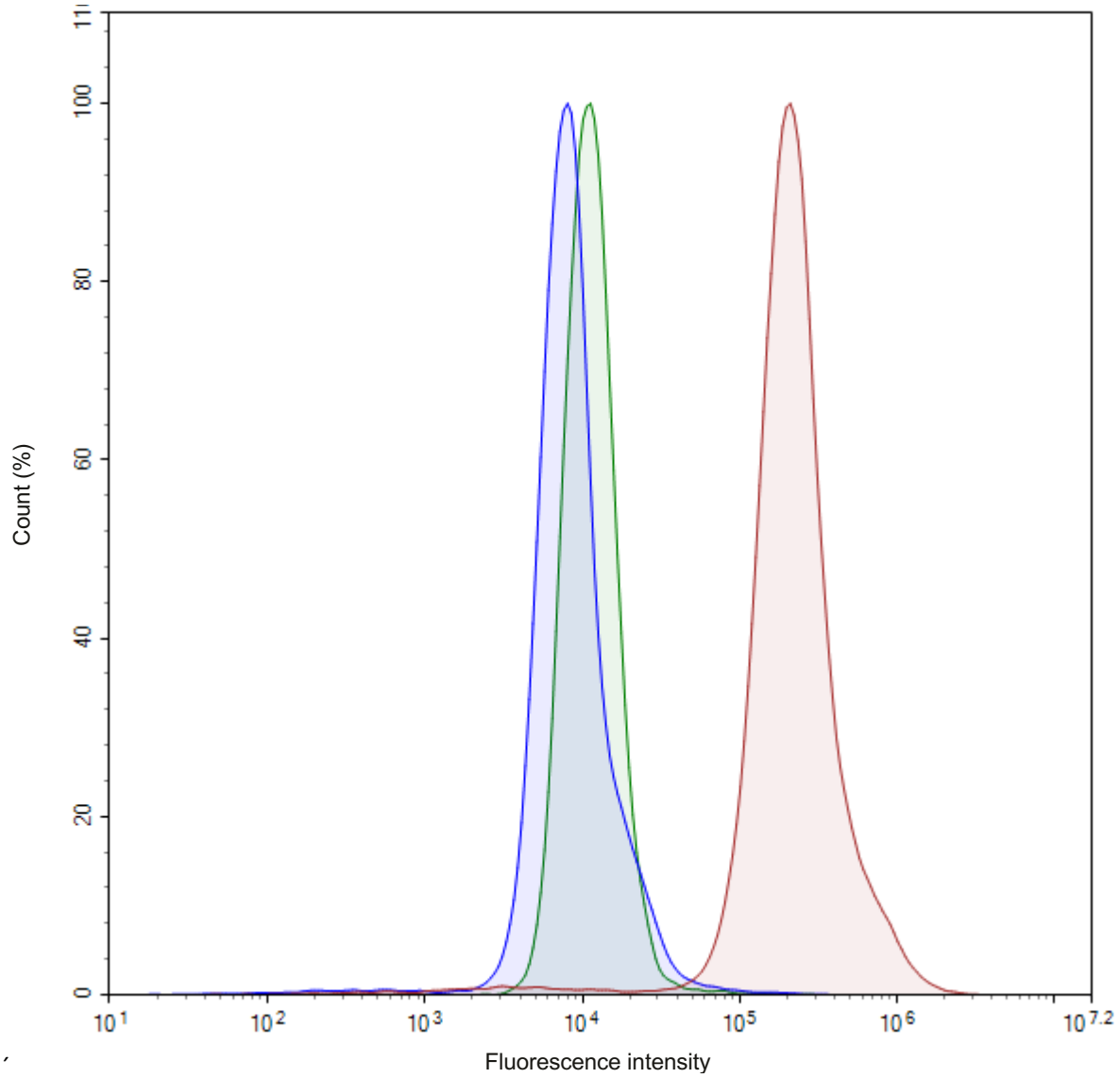


Figure S58. *Erb-Fab-DNA made with reagent **1** binding to EGFR on A431 EGFR+ positive cell surfaces analysed by flow cytometry. Blue: Untreated, Green: Competition, Red: Receptor binding. For the competition experiment, the cells were incubated with the unmodified Erbitux prior to incubation with Erb-Fab-DNA. The Erb-Fab-DNA showed binding capacity.*

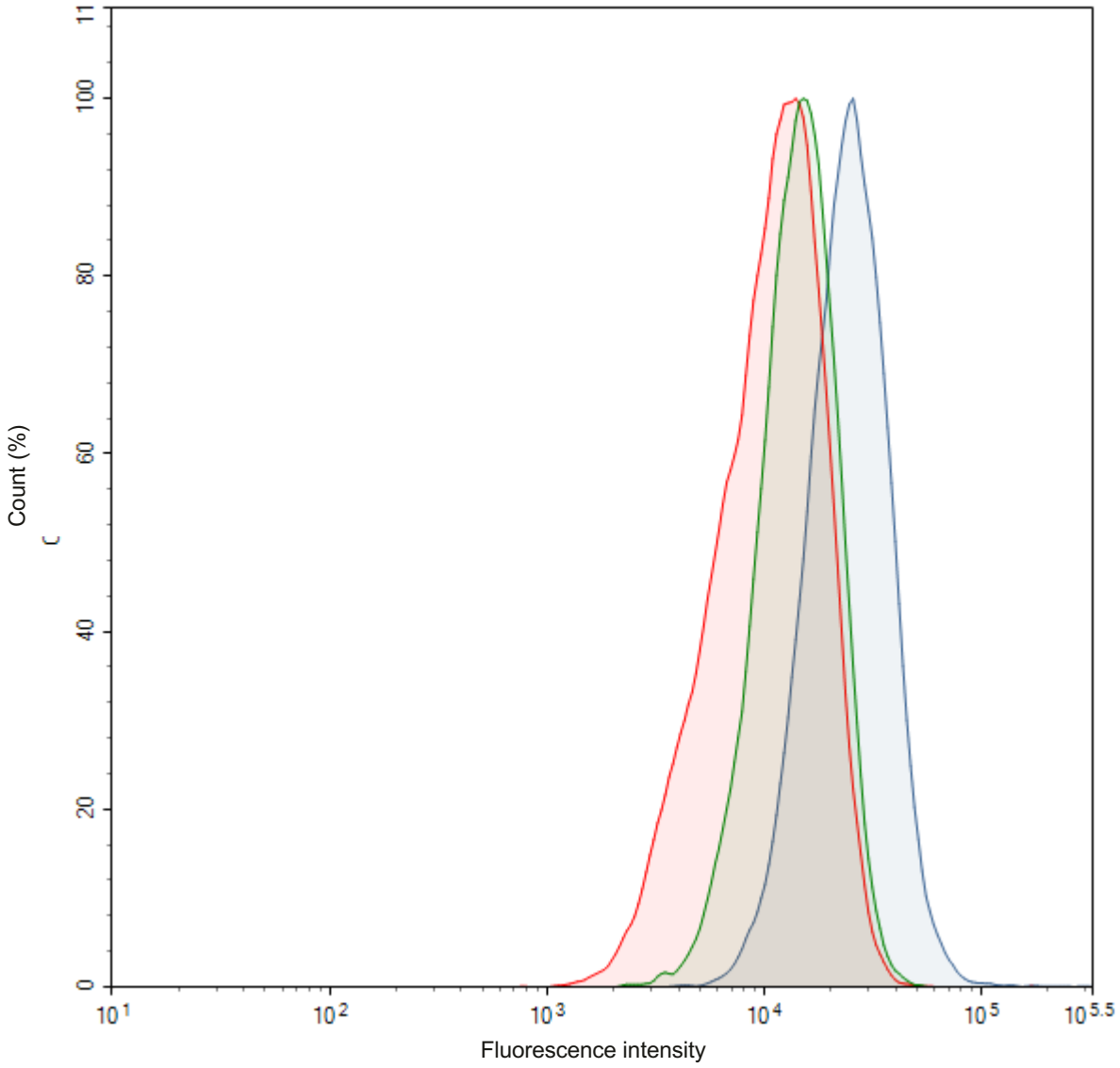


Figure S59. *Her-Fab-DNA made twith reagent **1** binding to Her2+ on SKBR3 Her2+ positive cell surfaces analysed by flow cytometry. Red: Untreated, Green: Competition, Blue: Receptor binding. For the competition experiment, the cells were incubated with the unmodified Herceptin prior to incubation with Her-Fab-DNA. The Her -Fab-DNA showed binding capacity.*

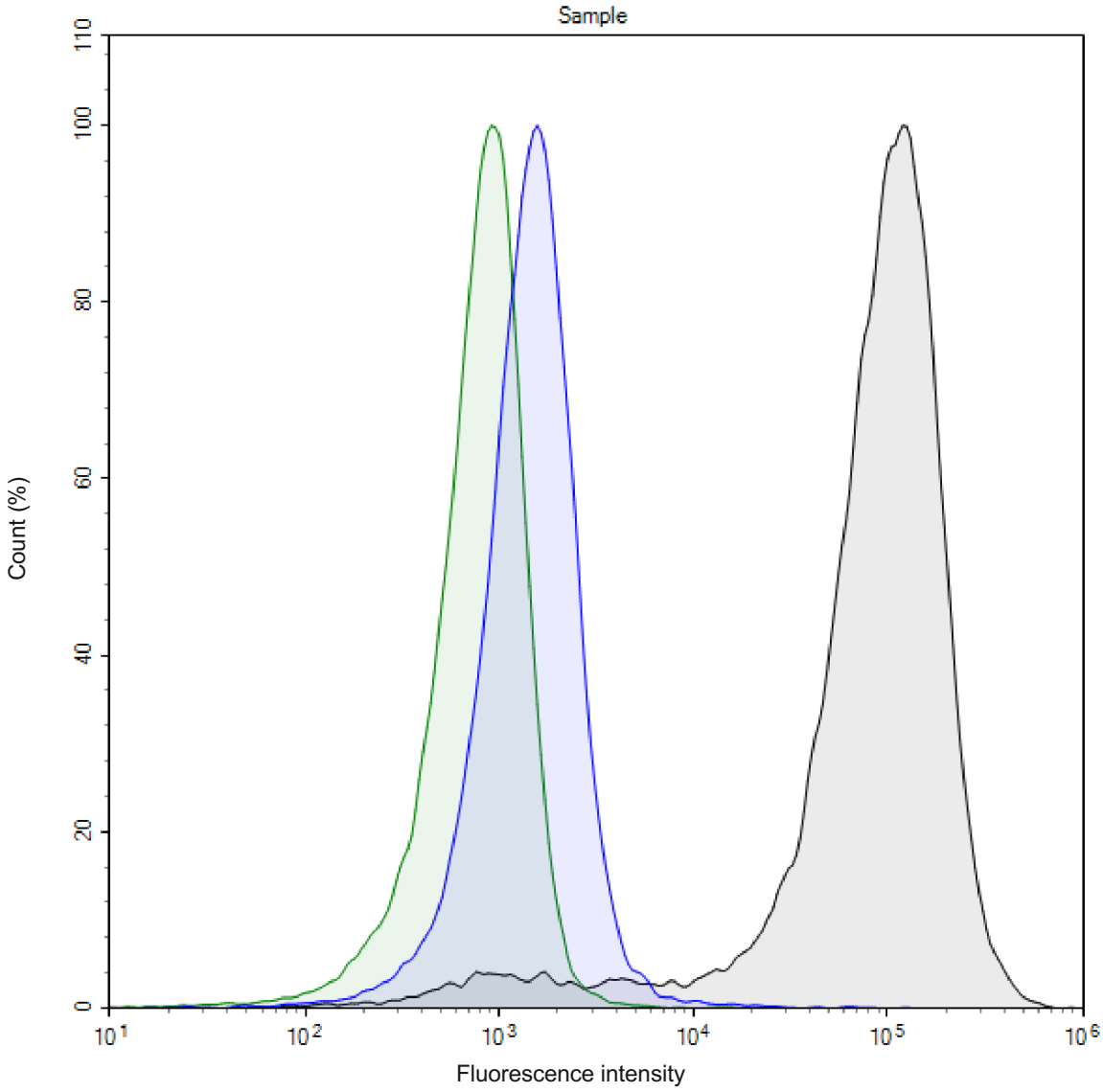


Figure S60. *Her-Fab-vivotag™ conjugate made through reagent* ***2*** *binding to Her2+ on SKBR3 Her2+ positive cell surfaces analysed by flow cytometry. For the competition assay, the cells were pre-treated with Herceptin. Green: Untreated, Blue: Competition, Black: Receptor binding. Her-Fab-vivotag™ showed binding capacity.*

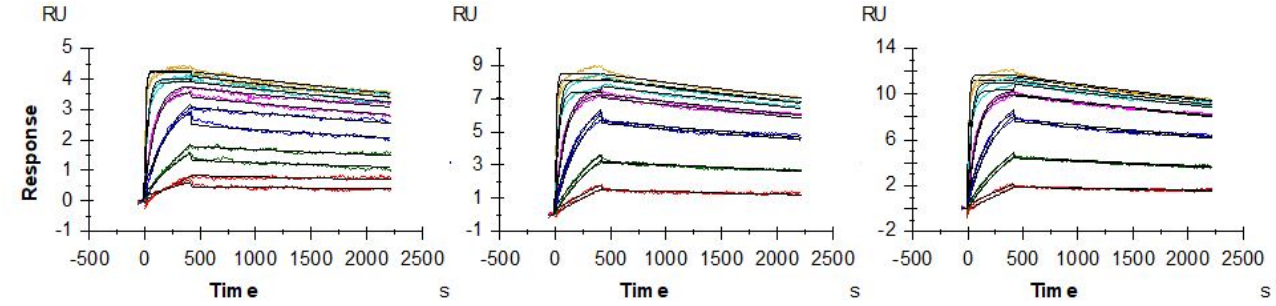
SPR

Methods:

The interaction of anti HER2 fab and modifications thereof to HER2 was characterised by SPR with a Biacore T200 (GE Healthcare) in HBS-EP+ running buffer using Biotin CAPture kit (GE HEalthcare #28920234). All steps were performed according to the manufacturer's instruction, with the exception that biotin capturing reagent was diluted to 5 µg/ml and injected for 25 s at 5ul/min. Biotinylated HER2 (ACROBiosciences #HE2-H82E2) was captured onto a Series S Sensor Chip CAP by injecting dilutions of 0.1-0.8 µg/ml HER2 in running buffer for 60s at 10 µl/min. Anti HER2 fab was injected at 6 different concentrations in a 1:3 serial dilution of 150 nM (modified with NEM conjugated thiol handle) or 5 concentrations dilution of 50 nM (PEG conjugates) in running buffer.

Comparison of Her-Fab-NEM-conjugate and native Her-Fab

a)



b)

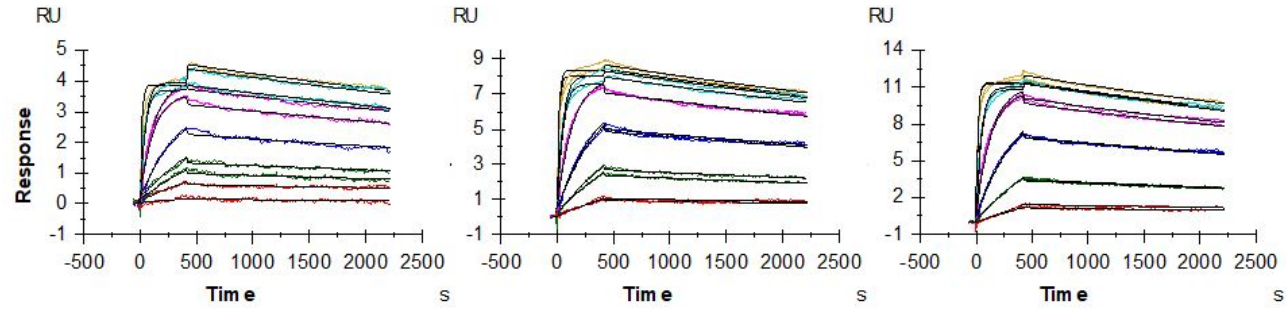


Figure S61. *The binding of Her-Fab, before and after modification with thiol handle, to HER2 captured at three different capture densities (14-35 RU) as monitored by SPR.* ***(a)*** *Binding curves of unmodified Her-Fab.* ***(b)*** *Binding curves of Her-Fab-NEM conjugate made by reagent* ***1****. Black curves plotted onto experimental data represent a Langmuir 1:1 binding model fit.*

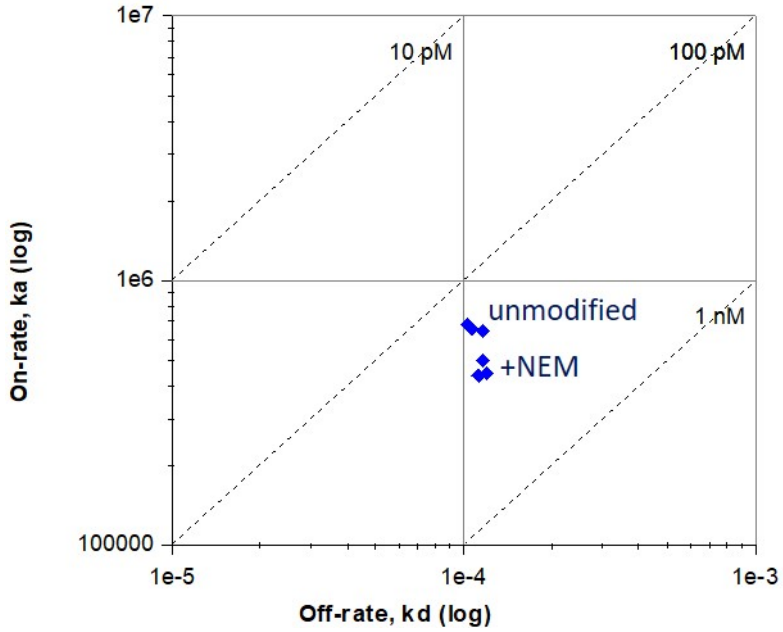


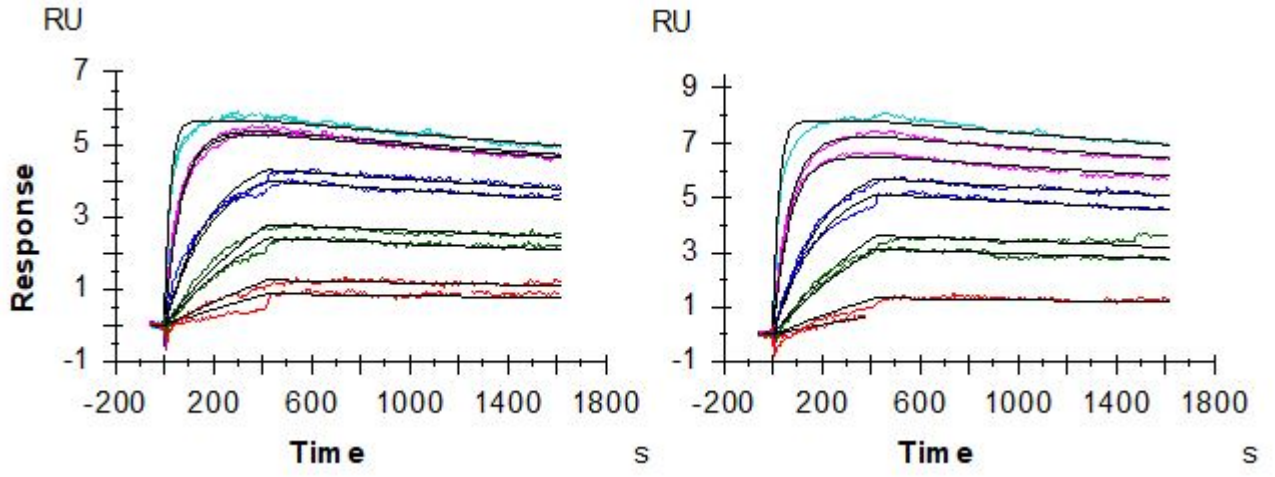
Figure S62. *Association rates plotted against dissociation rates of Her-Fab, before and after modification with thiol handle, binding to HER2 captured at different capture densities. Diagonal lines represent affinity (KD). The Her-Fab-NEM conjugates show no major difference in affinity.*

Sample	***Ligand*** *(µg/mL)*	***ka (1/Ms)***	***kd (1/s)***	***KD (M)***	***SE(ka) (1/Ms)***	***SE(kd) (1/s)***	***Chi² (RU²)***
	HER2 0.2	7×10^{5}	1×10^{-4}	2×10^{-10}	7×10^{2}	1×10^{-7}	5×10^{-3}
aHER2Fab	HER2 0.4	6×10^{5}	1×10^{-4}	2×10^{-10}	6×10^{2}	8×10^{-8}	1×10^{-2}
	HER2 0.8	6×10^{5}	1×10^{-4}	2×10^{-10}	5×10^{2}	8×10^{-8}	2×10^{-2}
	HER2 0.2	5×10^{5}	1×10^{-4}	2×10^{-10}	6×10^{2}	1×10^{-7}	6×10^{-3}
NEM	HER2 0.4	4×10^{5}	1×10^{-4}	3×10^{-10}	4×10^{2}	9×10^{-8}	1×10^{-2}
	HER2 0.8	4×10^{5}	1×10^{-4}	3×10^{-10}	4×10^{2}	8×10^{-8}	2×10^{-2}

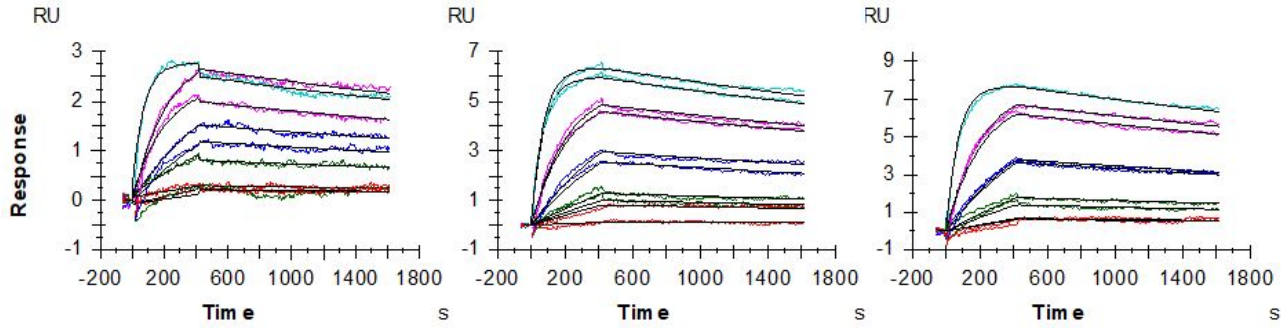
Table S1. *Kinetic parameters (ka, kd) and calculated affinity (KD) of Her-Fab before and after modification with thiol handle as determined by SPR.*

SPR of PEGylated Her-Fab

a)



b)



c)

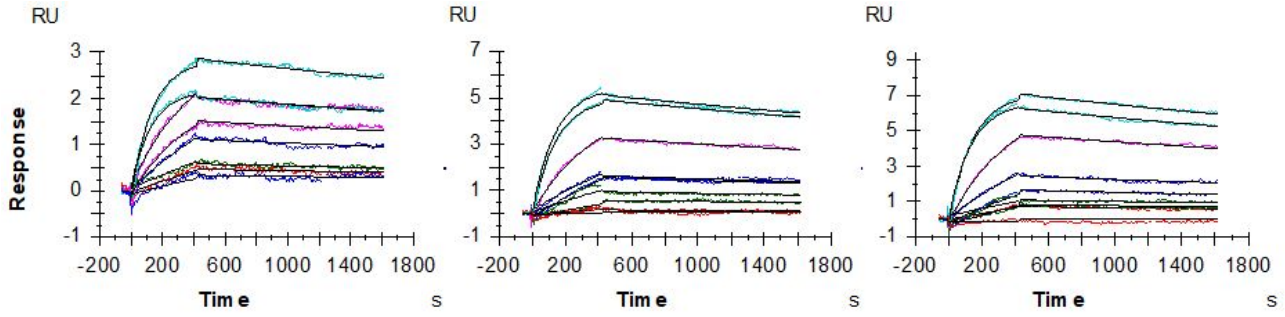


Figure S63. *The binding of Her-Fab (before and after modification with pseudo-cysteine handle and conjugation to PEG 5 kDa or 20 kDa) to HER2 captured at different capture densities (11-26 RU), as monitored by SPR.* ***(a)*** *Binding curves of unmodified Her-Fab.* ***(b)*** *Binding curves of Her-Fab-5 kDa PEG conjugate made by reagent* ***1****.* ***(c)*** *Binding curves of Her-Fab-20 kDa PEG conjugate made by reagent* ***1****. Black curves plotted onto experimental data represent Langmuir 1:1 binding model fit.*

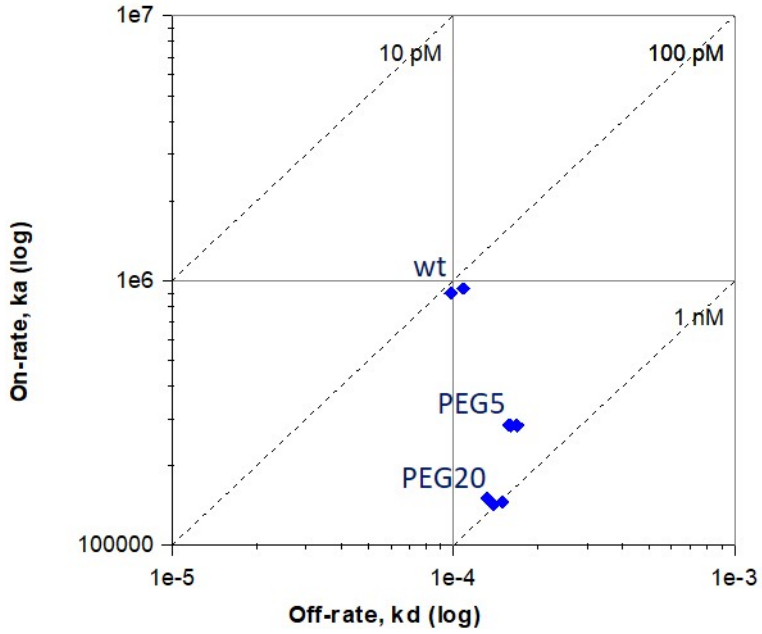


Figure S64. *Association rates plotted against dissociation rates of Her-Fab, before and after PEG modification, binding to HER2 captured at different capture densities. Diagonal lines represent affinity (KD). A 5-8 fold decrease in affinity was observed for Her-Fab-PEG conjugates.*

Sample	*Ligand (µg/ml)*	*ka (1/Ms)*	*kd (1/s)*	*KD (M)*	*SE(ka) (1/Ms)*	*SE(kd) (1/s)*	*Chi² (RU²)*
	HER2 0.2	9×10^{5}	1×10^{-4}	1×10^{-10}	9×10^{2}	2×10^{-7}	1×10^{-2}
aHER2Fab	HER2 0.4	9×10^{5}	1×10^{-4}	1×10^{-10}	1×10^{3}	2×10^{-7}	2×10^{-2}
	HER2 0.1	3×10^{5}	2×10^{-4}	6×10^{-10}	1×10^{3}	4×10^{-7}	5×10^{-3}
aHER2Fab-PEG5	HER2 0.2	3×10^{5}	2×10^{-4}	6×10^{-10}	3×10^{2}	2×10^{-7}	1×10^{-2}
	HER2 0.4	3×10^{5}	2×10^{-4}	6×10^{-10}	3×10^{2}	3×10^{-7}	2×10^{-2}
	HER2 0.1	1×10^{5}	1×10^{-4}	9×10^{-10}	4×10^{2}	4×10^{-7}	4×10^{-3}
aHER2Fab-PEG20	HER2 0.2	1×10^{5}	1×10^{-4}	1×10^{-9}	5×10^{2}	3×10^{-7}	7×10^{-3}
	HER2 0.4	1×10^{5}	1×10^{-4}	1×10^{-9}	3×10^{2}	3×10^{-7}	1×10^{-2}

Table S2. *Kinetic parameters (ka, kd) and calculated affinity (KD) of Her-Fab binding to different capture densities of HER2 before and after PEG modification as determined by SPR.*

Protein sequences

Ava-Fab

Heavy chain:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADF
KRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH

Light chain:

DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSG
TDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC

Ben-Fab

Heavy chain:

QVQLQQSGAEVKKPGSSVRVSCKASGGTFNNNAINWVRQAPGQGLEWMGGIIPMFGTAKYSQNFQ
GRVAITADESTGTASMELSSLRSEDTAVYYCARSRDLLLFPHHALSPWGRGTMVTVSSASTKGPSVF
PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
TQTYICNVNHKPSNTKVDKKVEPKSCDKTH

Light chain:

SSELTQDPAVSVALGQTVRVTCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSS
GNTASLTITGAQAEDEADYYCSSRDSSGNHWVFGGGTELTVLGQPKAAPSVTLFPPSSEELQANKA
TLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVT
HEGSTVEKTVAPTECS

Erb-Fab

Heavy chain:

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSR
LSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAASTKGPSVFPLAP
SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT
YICNVNHKPSNTKVDKKVEPKSCDKTH

Light chain:

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDF
TLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF
YPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC

Her-Fab

Heavy chain:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVK
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSASTKGPSVFP
LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
TQTYICNVNHKPSNTKVDKKVEPKSCDKTH

Light chain:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRS
GTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC

Rit-Fab

Heavy chain:

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKF
KGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSAASTKGPS
VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS
LGTQ TYICNVNHKP SNTKVDKKAE PKSCDKTH

Light chain:

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFSGSGSGT
SYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLN
NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLS
SPVTKSFNRGEC

Rem-Fab

Heavy chain:

EVKLEESGGGLVQPGGSMKLSCVASGFIFSNHWMNWVRQSPEKGLEWVAEIRSKSINSATHYAESV
KGRFTISRDDSKSAVYLQMTDLRTEDTGVYYCSRNYYGSTYDYWGQGTTLTVSSASTKGPSVFPLA
PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ
TYICNVNHKPSNTKVDKKVEPKSCDKTH

Light chain:

DILLTQSPAILSVSPGERVSFSCRASQFVGSSIHWYQQRTNGSPRLLIKYASESMSGIPSRFSGSGSGTD
FTLSINTVESEDIADYYCQQSHSWPFTFGSGTNLEVKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNN
FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC

Anti HIV Fab

Heavy chain:

QVQLVQSGAEVKKPGASVKVSCQASGYRFSNFVIHWVRQAPGQRFEWMGWINPYNGNKEFSAKF
QDRVTFTADTSANTAYMELRSLRSADTAVYYCARVGPYSWDDSPQDNYYMDVWGKGTTVIVSSA
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTH

Light chain:

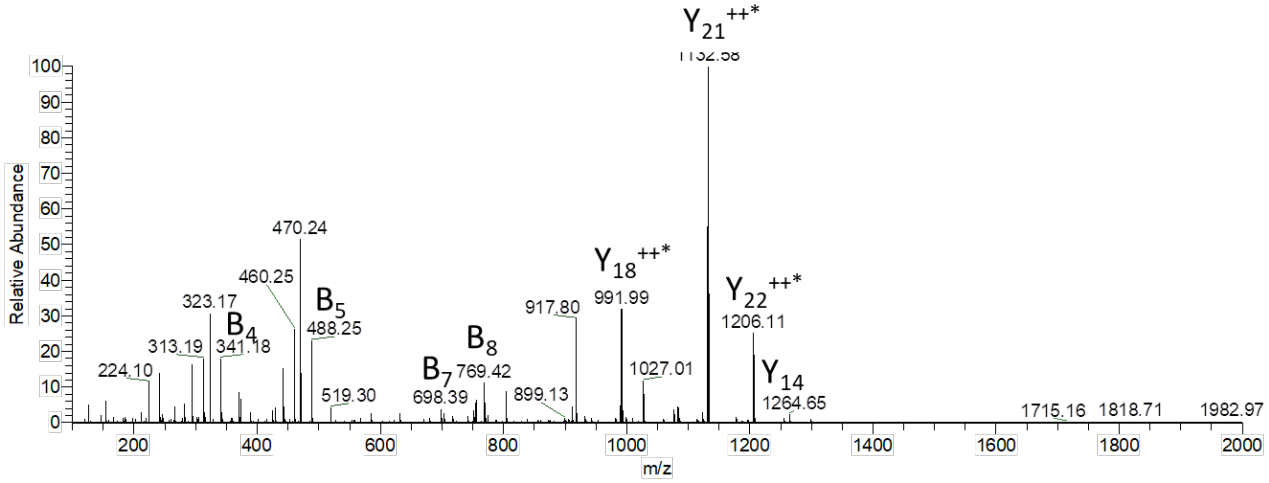
EIVLTQSPGTLSLSPGERATFSCRSSHSIRSRRVAWYQHKPGQAPRLVIHGVSNRASGISDRFSGSGSG
TDFTLTITRVEPEDFALYYCQVYGASSYTFGQGTKLERKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
RSPVTKSFNRGEC

MS/MS of NEM-modified conjugates

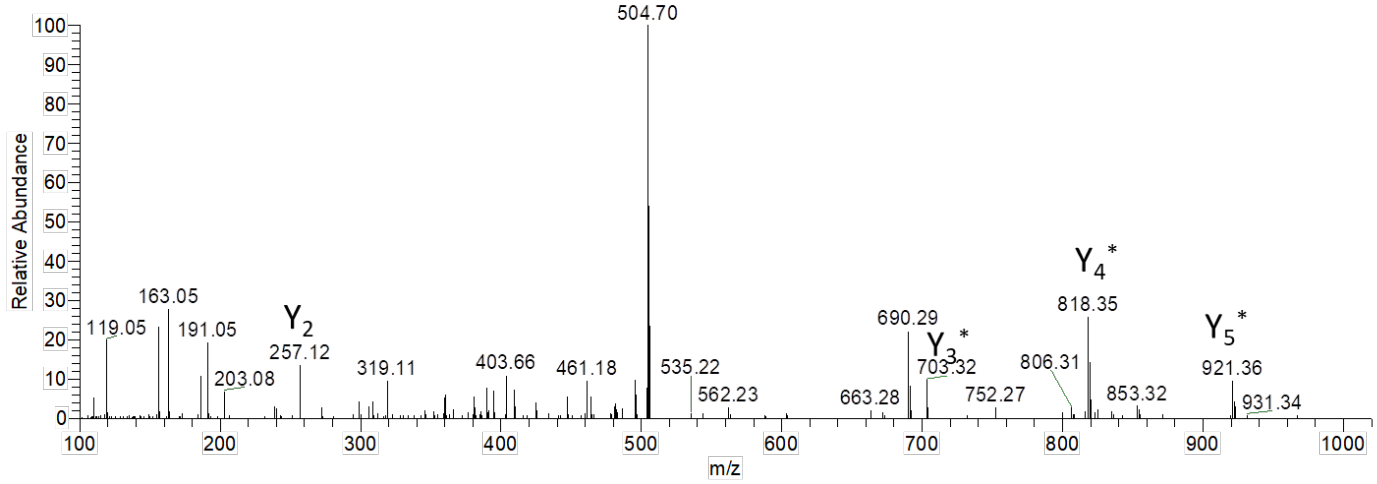
Unreduced and reduced + iodoacetamide alkylated conjugates of Her-Fab/hGH with NEM were digested with SMART-Trypsin according to the suppliers' instructions.[a] The resulting digests were analysed by a Waters UPLC connected to a Thermo Orbitrap Fusion using the Thermo Xcalibur software.

Example of Her-Fab reagent 1 NEM conjugate analysis:

K-136 AA125-150 (HC): GPSVFPLAPSSK*STSGGTAALGCLVK



K-225 AA222-227 (HC): SCDK*TH



[a] Thermo Scientific "Smart Digest Kit" part No. 60109.

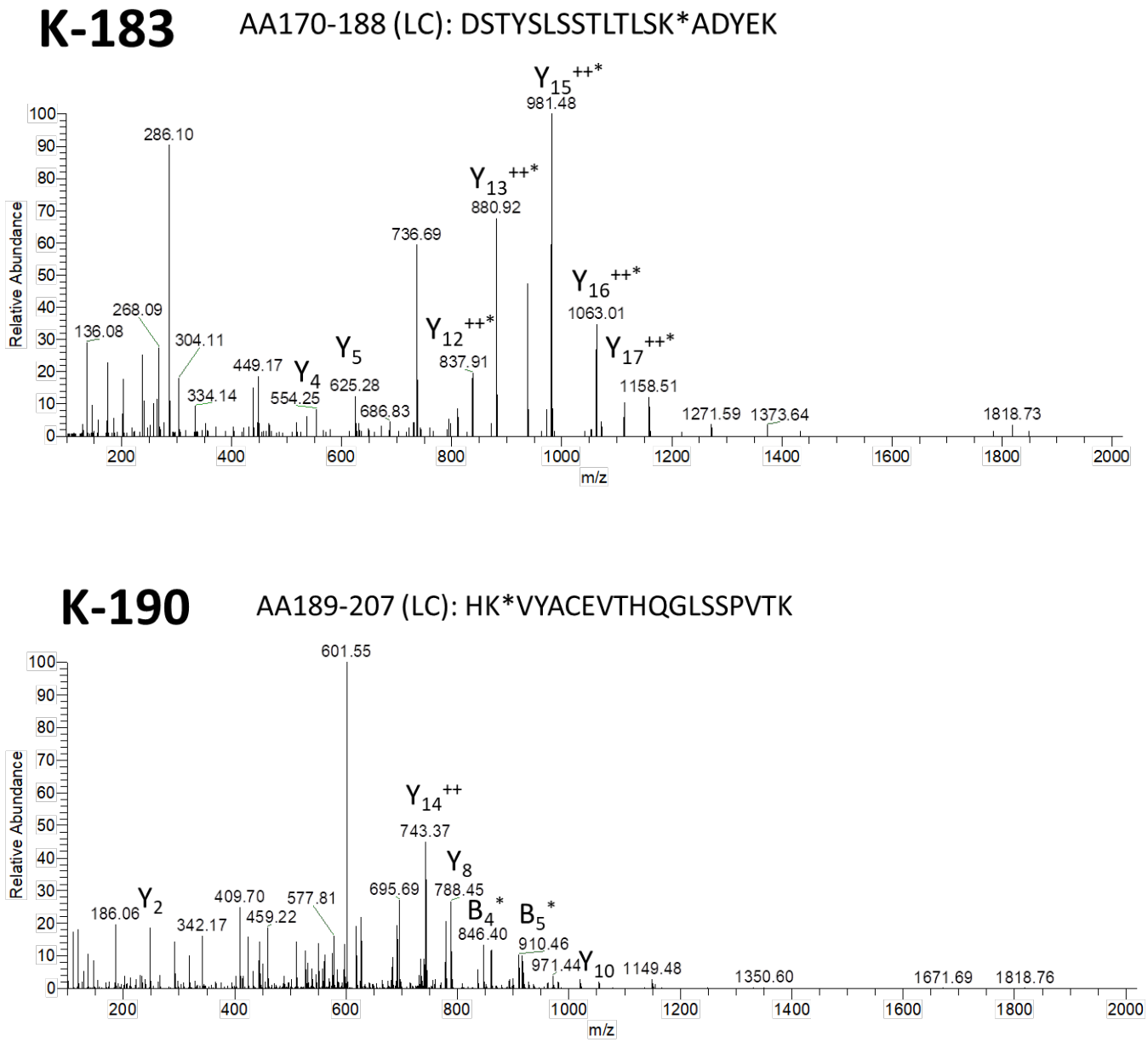


Figure S65. *Modifications of Her-Fab made through reagent* ***1*** *analysed by MSMS. The +318-modification corresponding to the installed thiol handle with NEM is indicated by and asterisk. The peptides containing K136 (HC), K225 (HC), 183 (LC), and K190 (LC) were identified as hits. The peptide containing K225 occasionally showed as a K221 hit. The K221 and K225 were never found simultaneously.*

hGH reagent **2** NEM conjugate

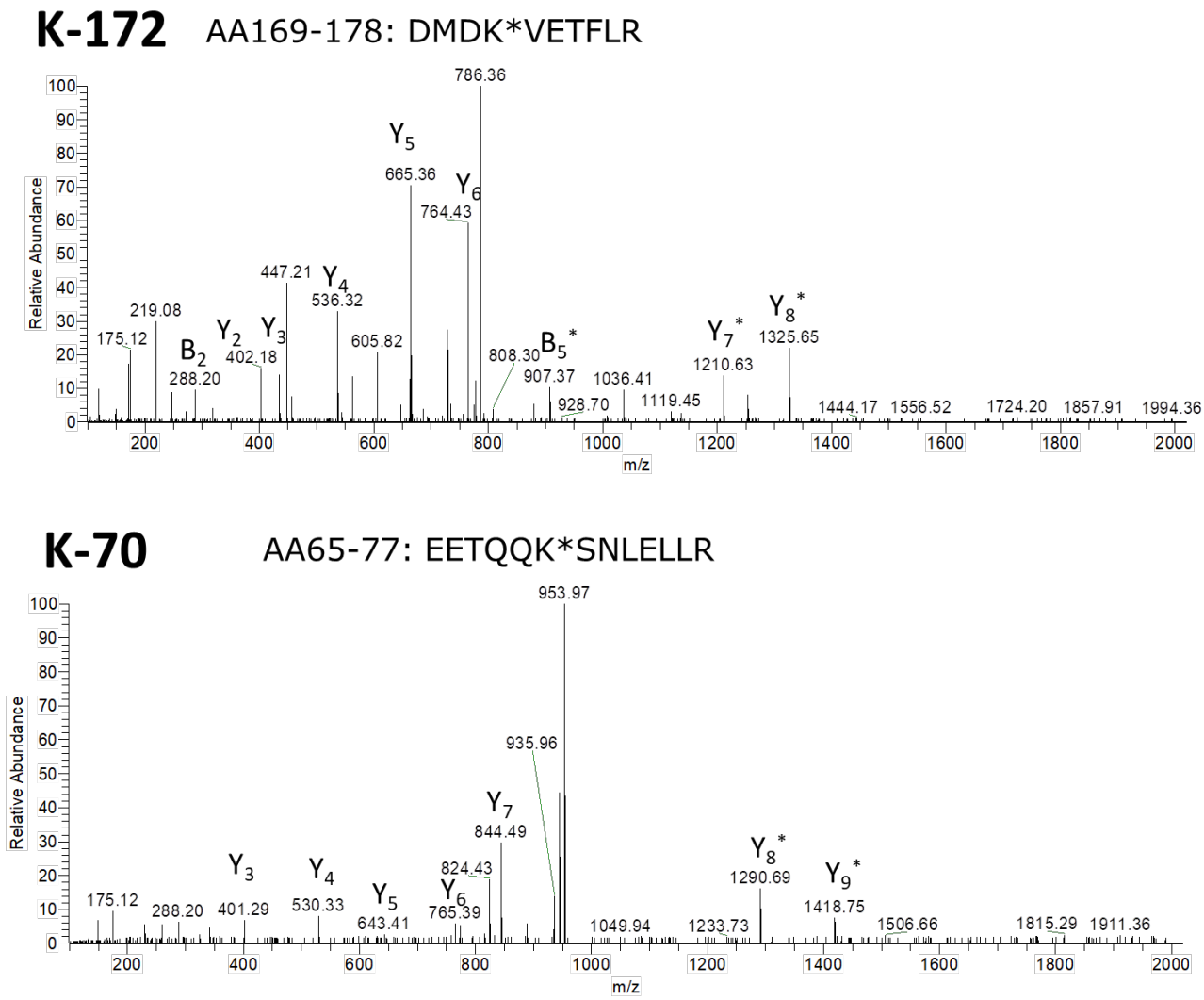


Figure S66. *Modifications of hGH made through reagent **2** analysed by MSMS. The +318-modification corresponding to the installed thiol handle with NEM is indicated by and asterisk. The peptides containing K172 and K70 were identified as hits.*

Comparison of the reagents

The reagents **1**, **2**, and **3** were compared for the labelling of Her-Fab. No differences were observed between them on Her-Fab with regards to labelling pattern. The conjugates were digested by AspN or Trypsin obtained through reagent **1**, **2**, and **3**.

Residue	***Reagent 1***		***Reagent 2***		***Reagent 3***	
	Trypsin	AspN	Trypsin	AspN	Trypsin	AspN
HC: K136	1.1×10^9	2.2×10^9	2.1×10^9	3.7×10^9	2.3×10^9	2.7×10^9
LC: K190*	6.6×10^8	-	1.1×10^9	-	1.1×10^9	4.8×10^8
LC: K207*	3.9×10^8	-	6.7×10^8	-	8.4×10^8	-
HC: K221¤	1.4×10^8	3.4×10^8	1.4×10^8	4.8×10^8	2.7×10^8	3.7×10^8
LC: K183	9.3×10^7	3.2×10^2	5.0×10^7	3.0×10^8	8.0×10^7	3.1×10^8

Table S3. *Relative intensities of the modified lysines on Her-Fab after reactions through reagent **1**, **2**, or **3** as analysed by peptide mapping following trypsin or AspN digestion. *: all sites within the same peptide when digested with AspN (AA 185-214). ¤: both K221 and K225 showed as hits during repeated digestions, albeit never at the same time.*

Residue	***Reagent 2***	***Reagent 3***
K172	2.5×10^9	7.5×10^8
K70	3.5×10^8	2.5×10^7

Table S4. *Relative intensities of the modified lysines on hGH as analysed by peptide mapping following trypsin digestion.*

MS/MS hits of all Fabs

The NEM conjugates of the panel of Fabs were all analysed as Her-Fab and all hits have been collected in the following figures. Generally, the hits were similar between the Fabs.

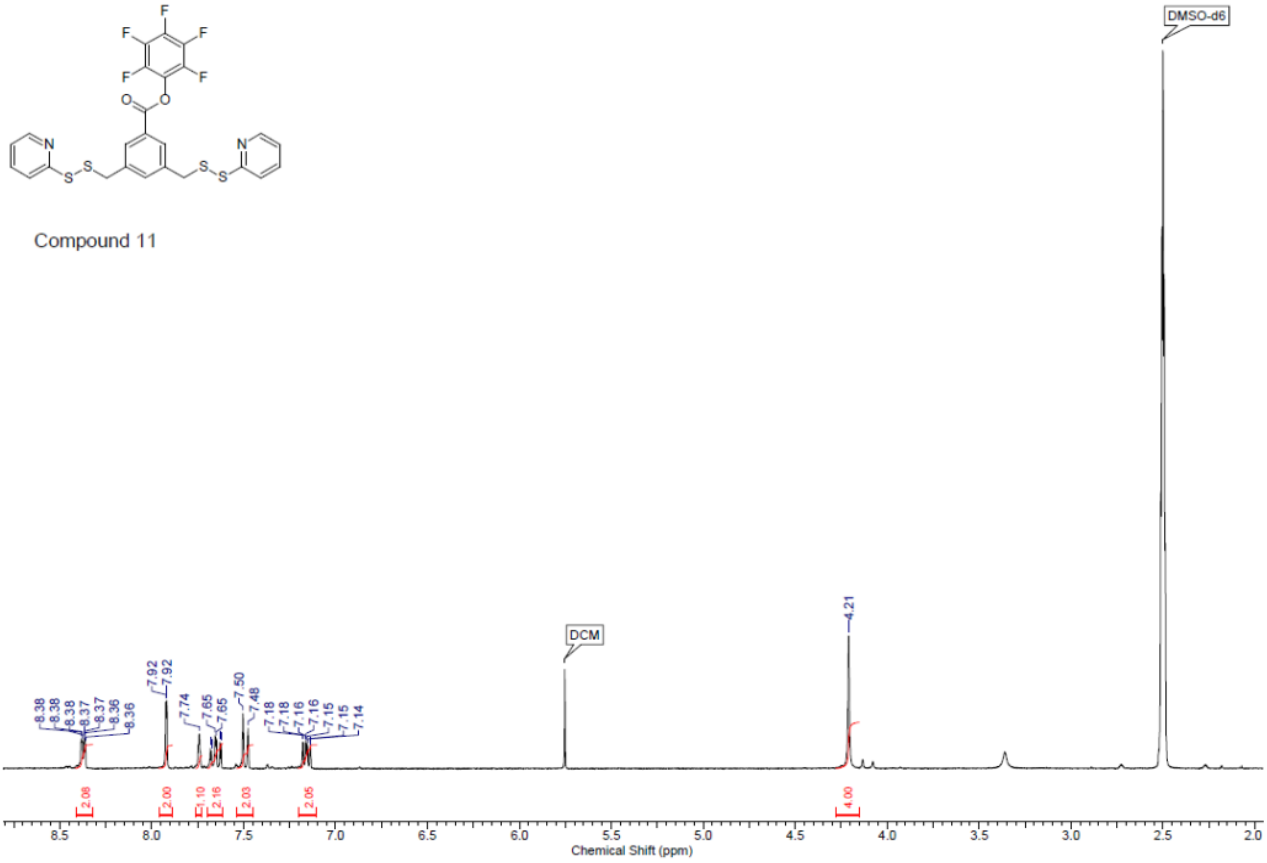
Hits identified for Heavy chains of all Fabs

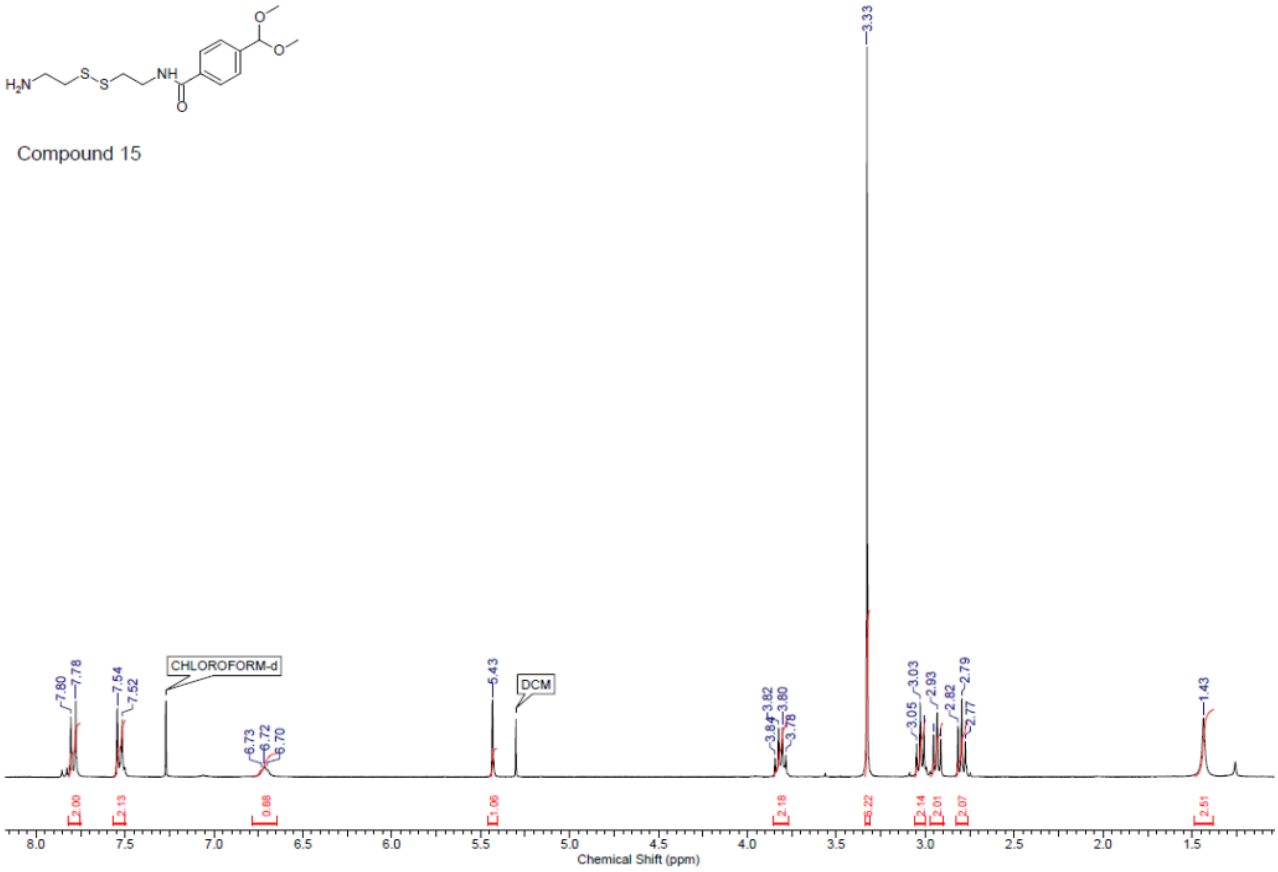
Figure S67. *Sequence alignment of heavy chains between ava-,ben-,-erb-, her-,rem-, rit-, and aHIV-Fabs. Hits obtained by MS/MS are indicated across the sequences in red, with the corresponding residues indicated in green on the aHIV-fab sequence. The alignment was made using the Clustal Omega program available from https://www.ebi.ac.uk/Tools/msa/clustalo/*

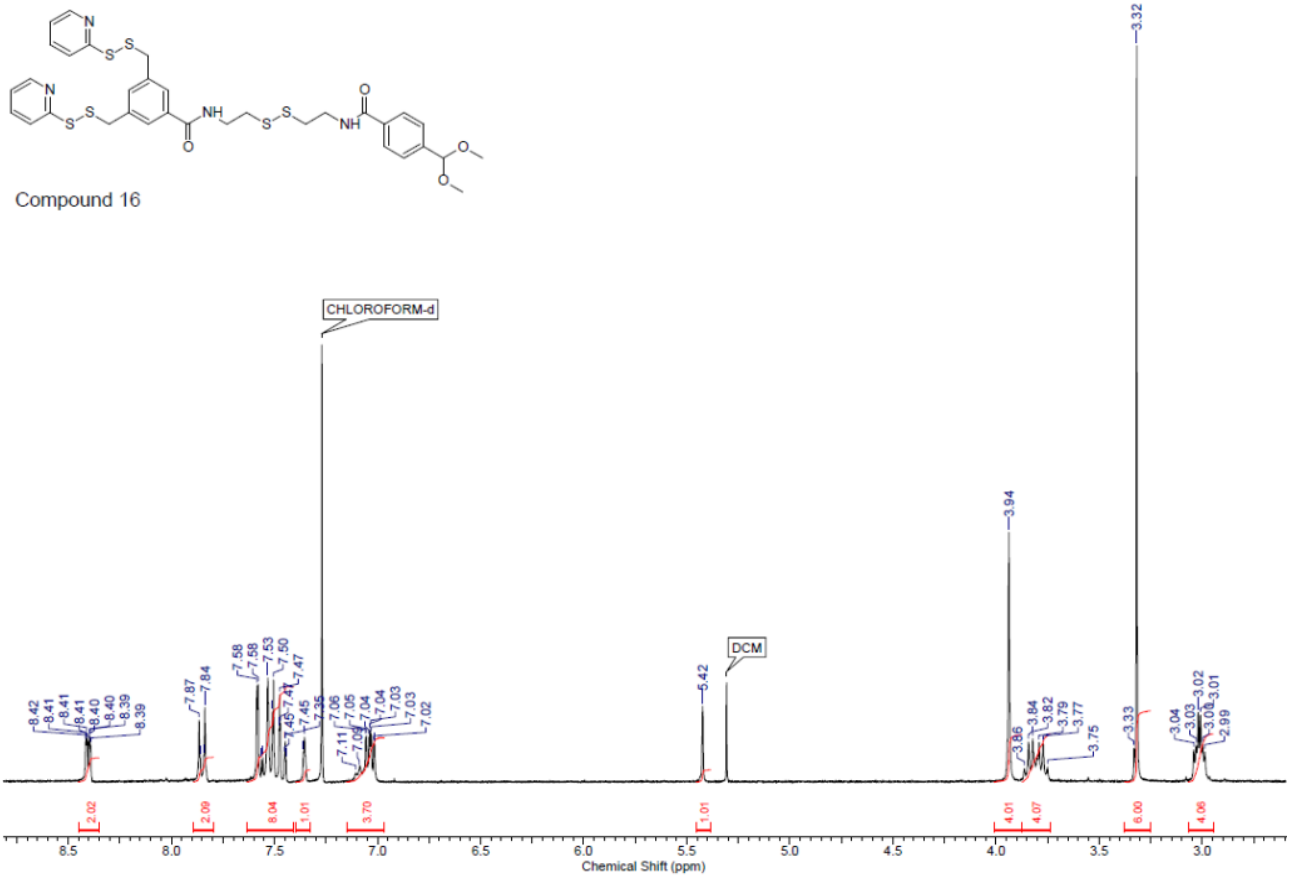
Hits identified on Light chains for all Fabs

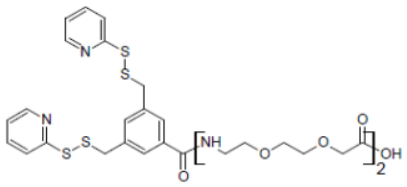
Figure S68. *Sequence alignment of light chains between ava-,ben-,-erb-, her-,rem-, rit-, and aHIV-Fabs. Hits obtained by MS/MS are indicated across the sequences in red, with the corresponding residues indicated in green on the aHIV-Fab sequence. The alignment was made using the Clustal Omega program available from https://www.ebi.ac.uk/Tools/msa/clustalo/*

1HNMR Spectra of Synthesized Intermediate Compounds

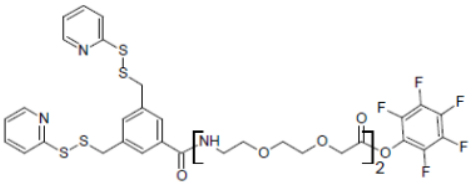



Compound 15
CHLOROFORM-d
DCM
Chemical Shift (ppm)

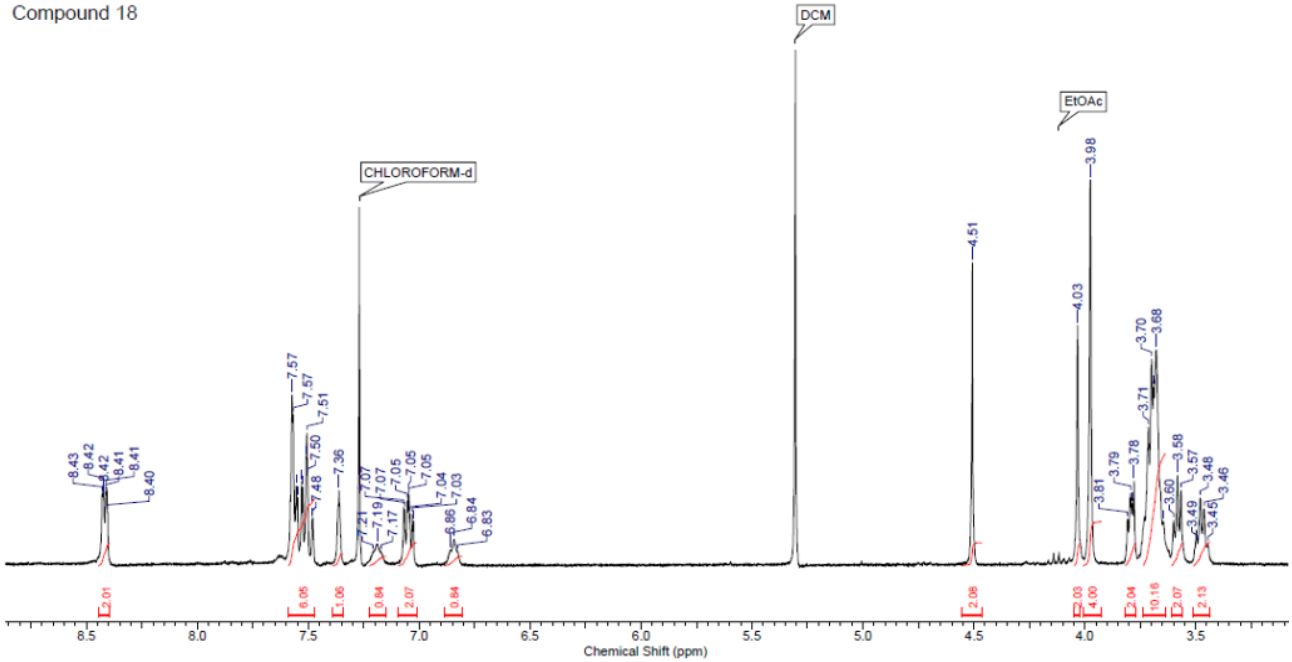

Compound 16
CHLOROFORM-d
DCM
Chemical Shift (ppm)

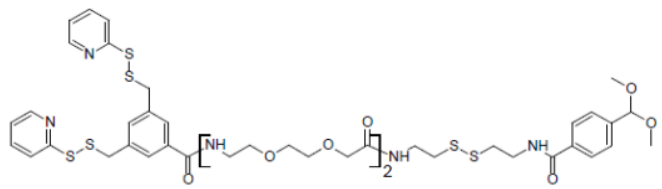


Compound 17

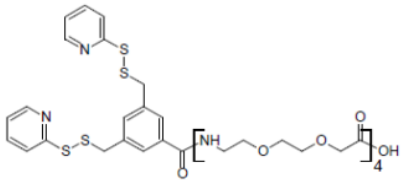


Compound 18

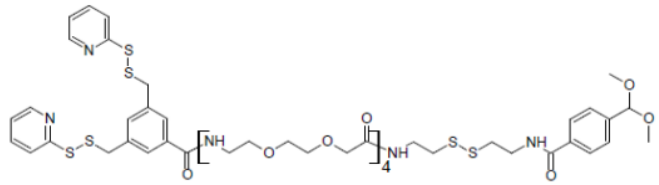




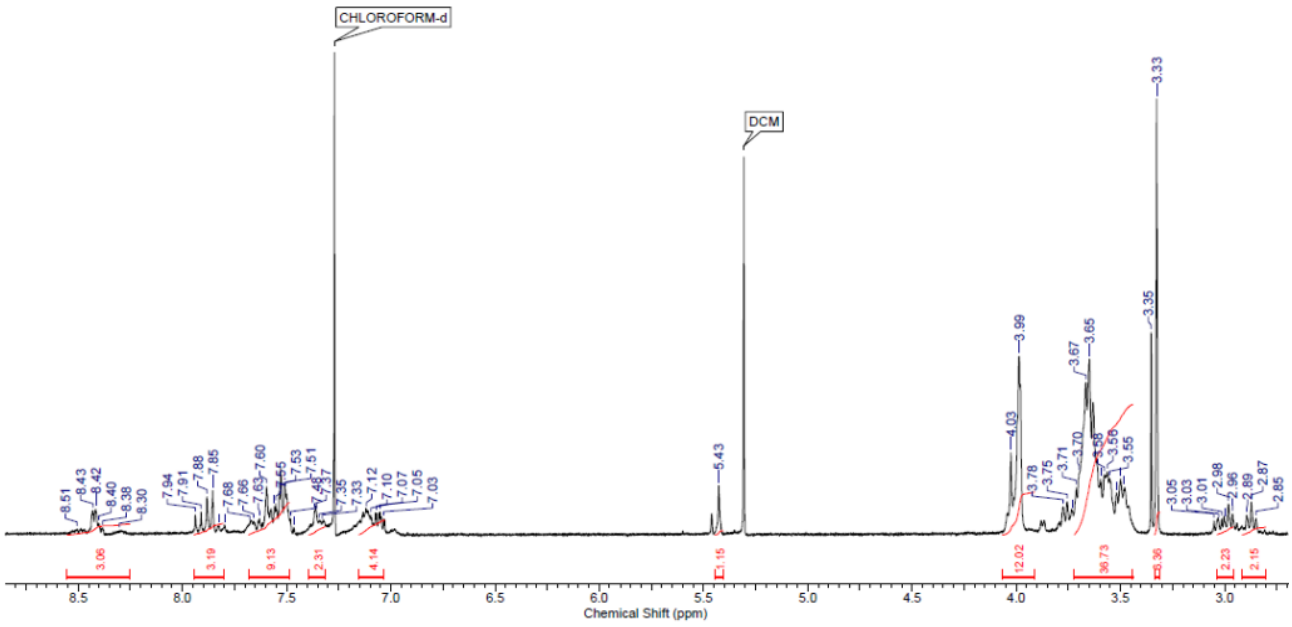
Compound 19



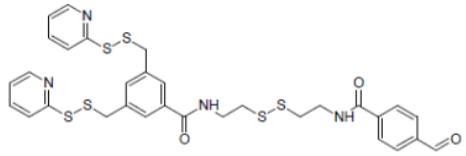
Compound 20



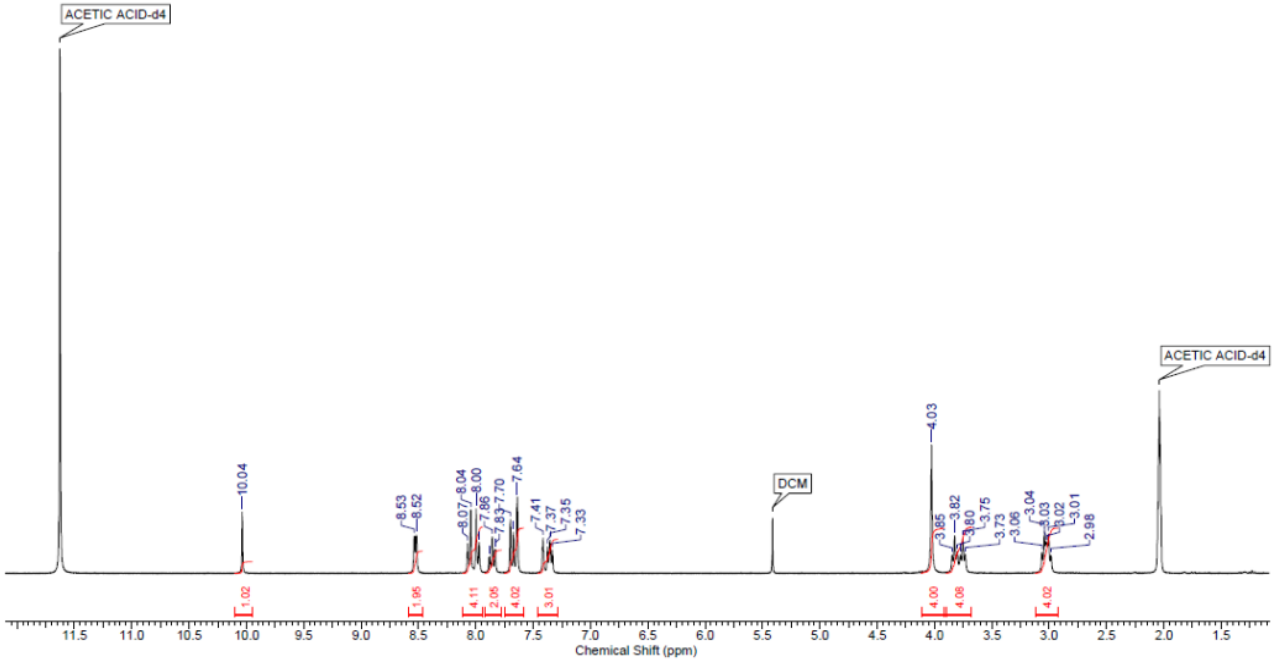
Compound 21

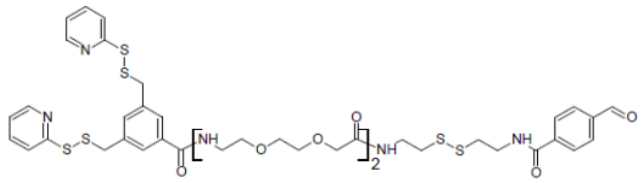


1HNMR Spectra of Final Reagents

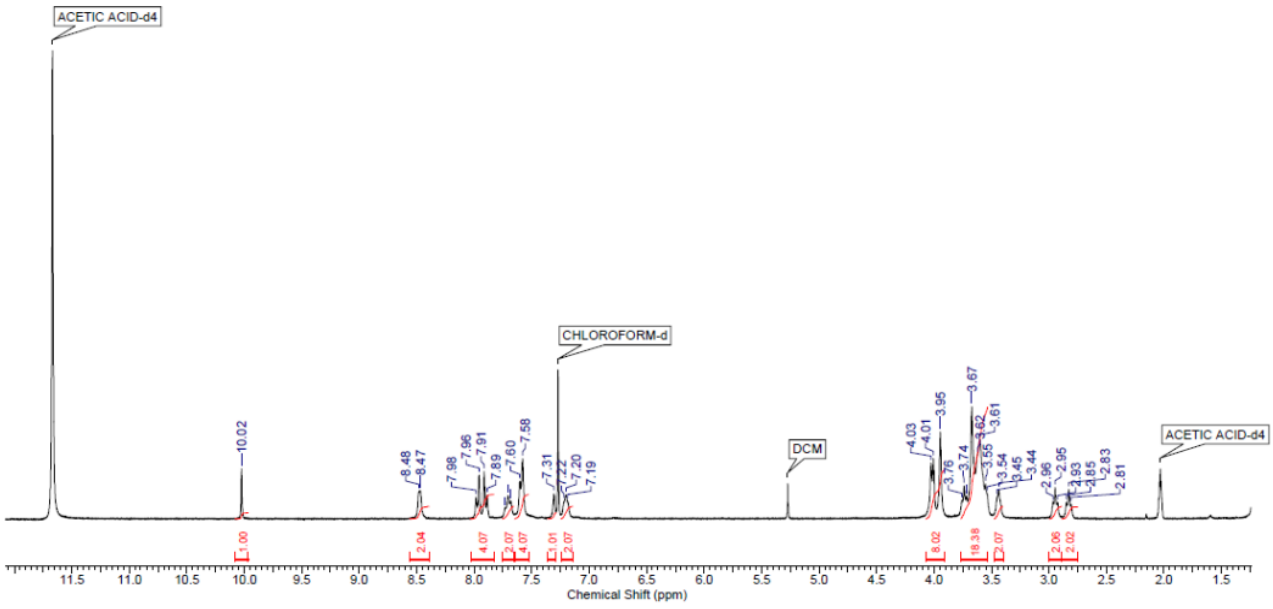


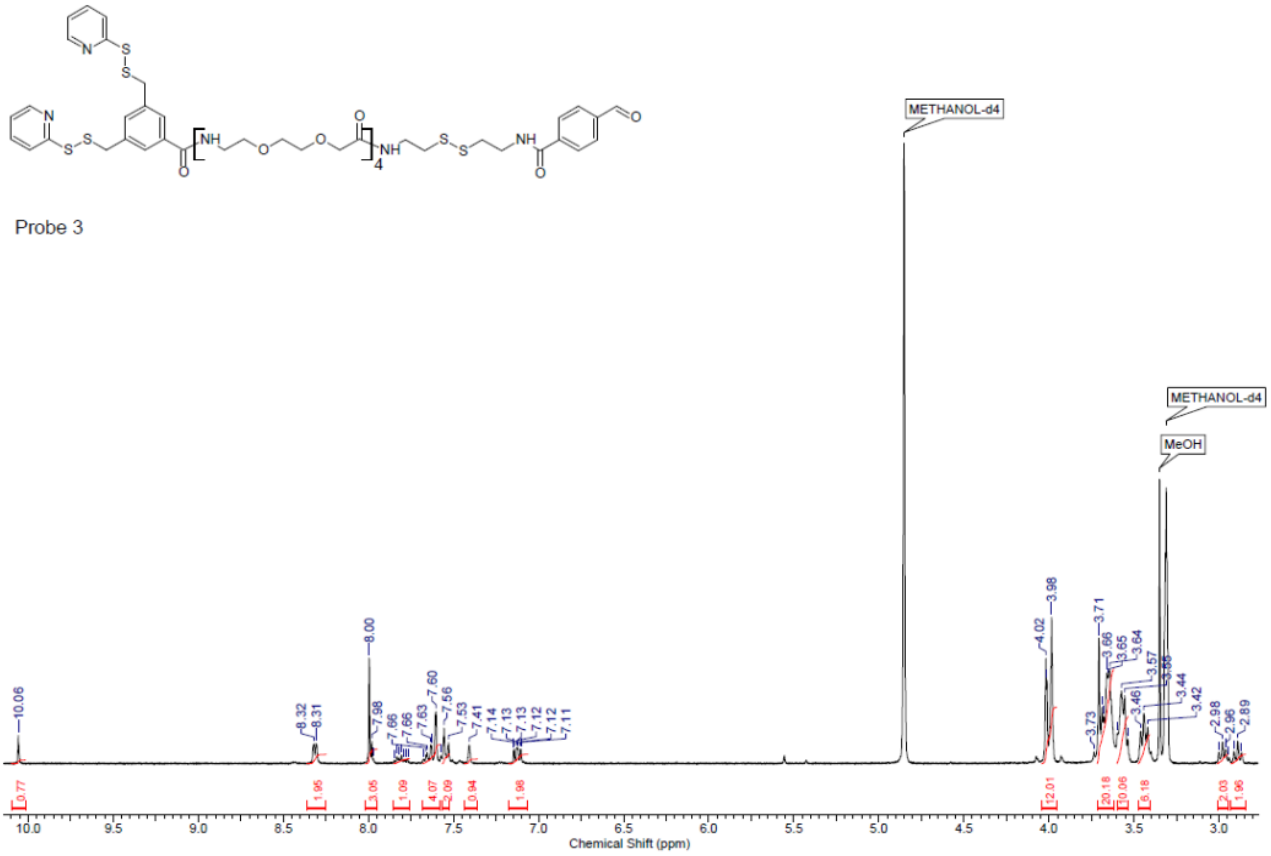
Probe 1





Probe 2




Probe 3
METHANOL-d4
METHANOL-d4
MeOH
10.06
8.32
8.31
8.00
7.98
7.66
7.66
7.63
7.60
7.56
7.53
7.41
7.14
7.13
7.13
7.12
7.12
7.11
4.02
3.98
3.73
3.71
3.66
3.65
3.64
3.57
3.46
3.44
3.42
2.98
2.96
2.89
0.77
1.95
3.05
1.09
4.07
2.09
0.94
1.98
2.01
0.18
0.06
5.18
0.03
1.96
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
Chemical Shift (ppm)

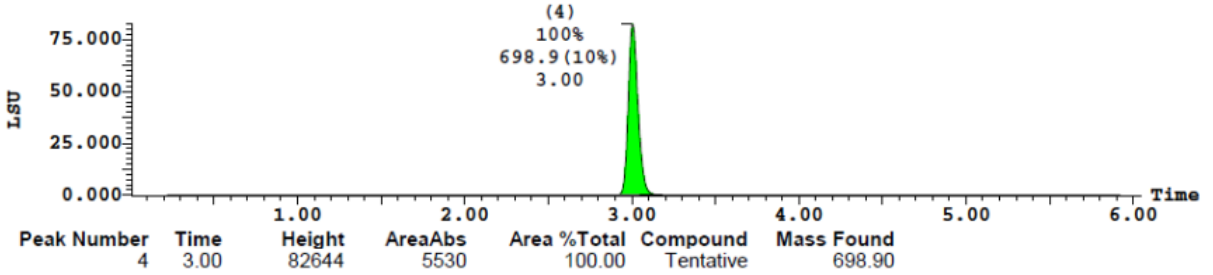
LC-MS of Final Reagents **1** - **3**

Openlynx Report - Page 1

Sample name:ZC-307-025-1a Probe 1 Vial:5,3:2,E Date:30-Jan-2019
Sample Number: 312 Description:1208 Time:13:16:24

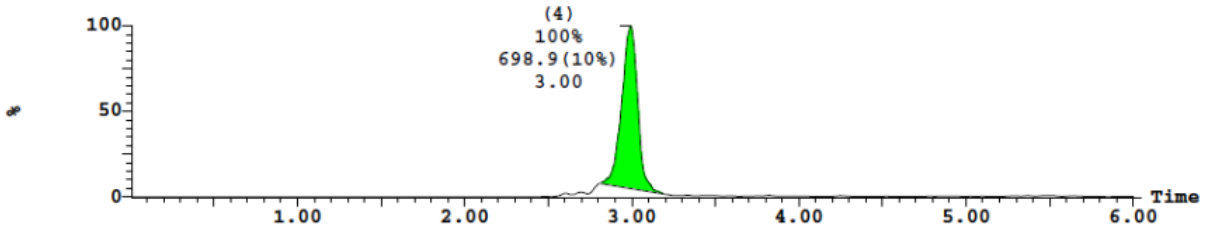
Sample: 312 File: ZC-307-025-1a Column: Kinetex C18, 5µm 4.6x50

(1) ELSD Signal 82.731
Range: 82.730

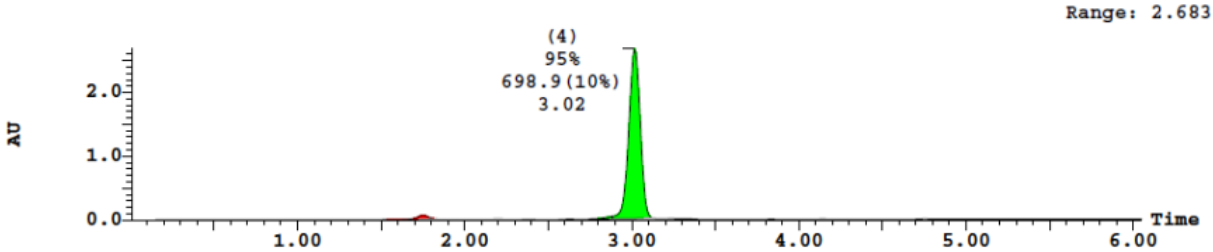


Peak Number	Time	Height	AreaAbs	Area %Total	Compound	Mass Found
4	3.00	82644	5530	100.00	Tentative	698.90

1: MS ES+ :721.9+699.9 2.3e+006



2: UV Detector: 254 2.683
Range: 2.683

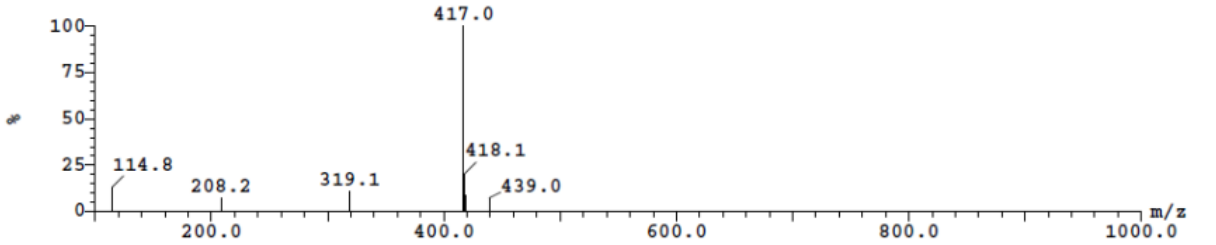


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		1.75	6967	3.10	0	68782	
2		2.20	1361	0.60	0	14480	
3	Tentative	2.88	2398	1.07	0	40691	698.90
4	Tentative	3.02	213052	94.71	0	2663521	698.90
5		3.32	545	0.24	0	6488	
6		4.14	626	0.28	0	7558	

Sample name:ZC-307-025-1a Vial:5,3:2,E Date:30-Jan-2019
Sample Number: 312 Description:1208 Time:13:16:24

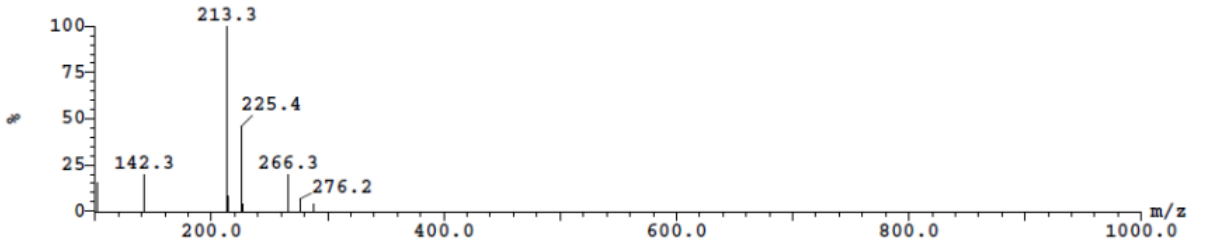
Peak ID	Compound	Time	Mass Found
1		1.75	

1:(Time: 1.75) 1:MS ES+ 1.1e+006



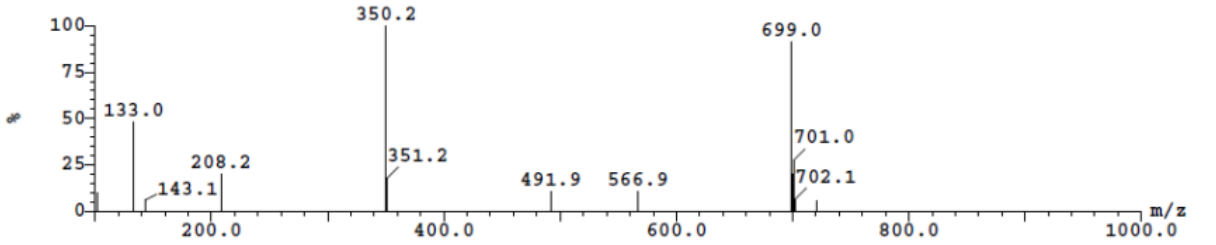
Peak ID	Compound	Time	Mass Found
2		2.20	

2:(Time: 2.20) 1:MS ES+ 6.1e+005



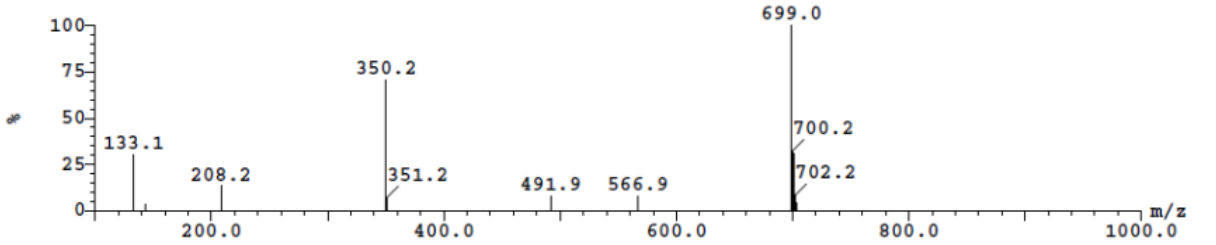
Peak ID	Compound	Time	Mass Found
3	Tentative	2.88	698.90

3:(Time: 2.88) 1:MS ES+ 1.9e+006



Peak ID	Compound	Time	Mass Found
4	Tentative	3.00	698.90

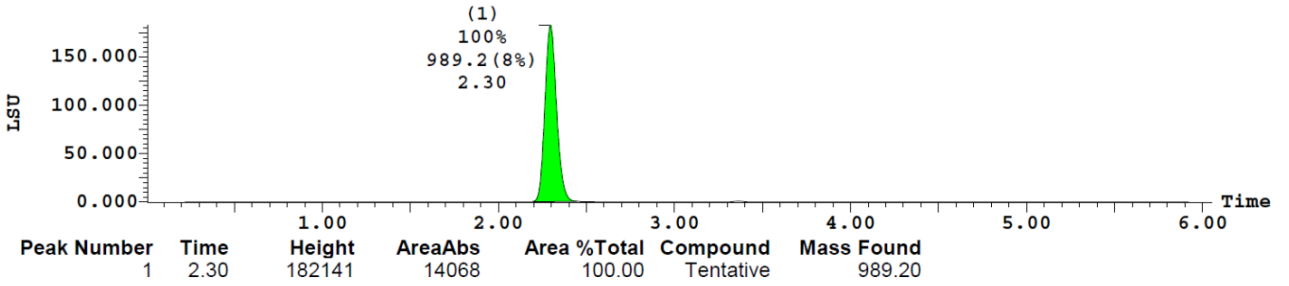
4:(Time: 3.00) 1:MS ES+ 6.9e+006



Sample name:ZC-297-033-2 Probe 2 Vial:5,3:1,E Date:12-Jun-2018
Sample Number: 103 Description:1134 Time:11:06:48

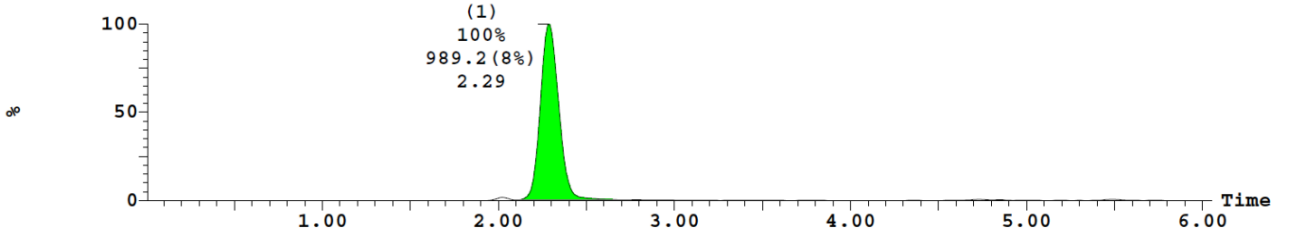
Sample: 103 File: ZC-297-033-2 Column: Kinetex C18, 5µm 4.6x50

(1) ELSD Signal 182.375
Range: 182.375

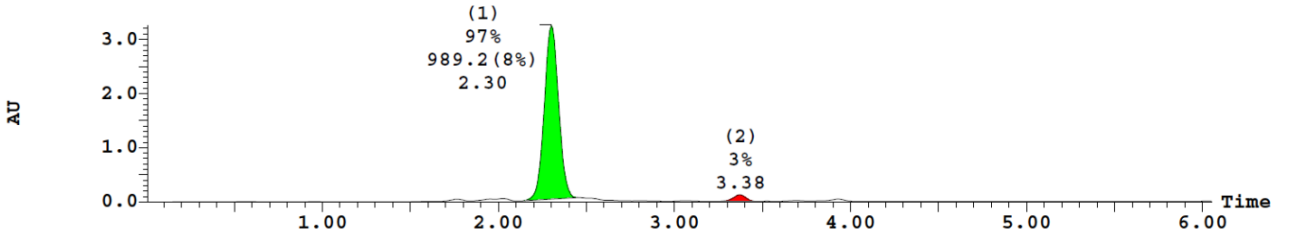


Peak Number	Time	Height	AreaAbs	Area %Total	Compound	Mass Found
1	2.30	182141	14068	100.00	Tentative	989.20

1: MS ES+ :1012.2+990.2 1.5e+007



2: UV Detector: 254 3.258
Range: 3.258

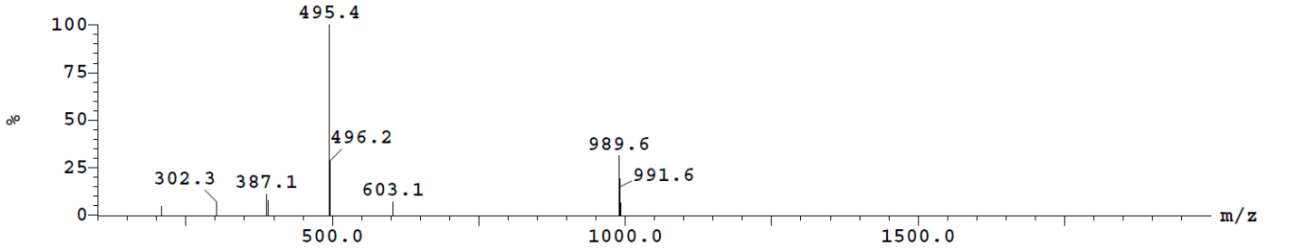


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Tentative	2.30	302214	96.85	0	3208955	989.20
2		3.38	9841	3.15	0	119760	

Sample name:ZC-297-033-2 Vial:5,3:1,E Date:12-Jun-2018
Sample Number: 103 Description:1134 Time:11:06:48

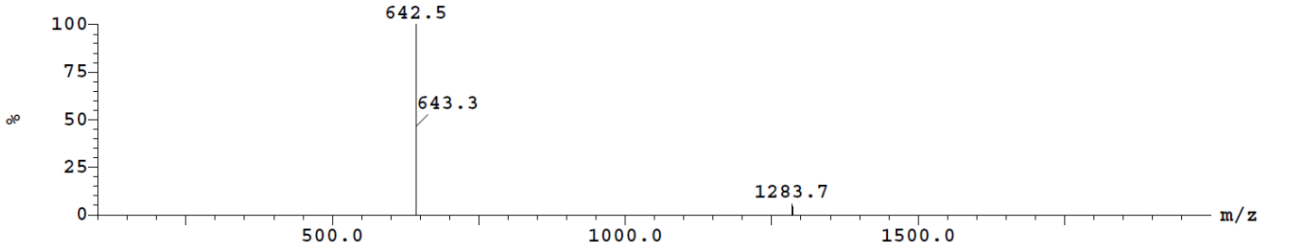
Peak ID	Compound	Time	Mass Found
1	Tentative	2.29	989.20

1:(Time: 2.29)



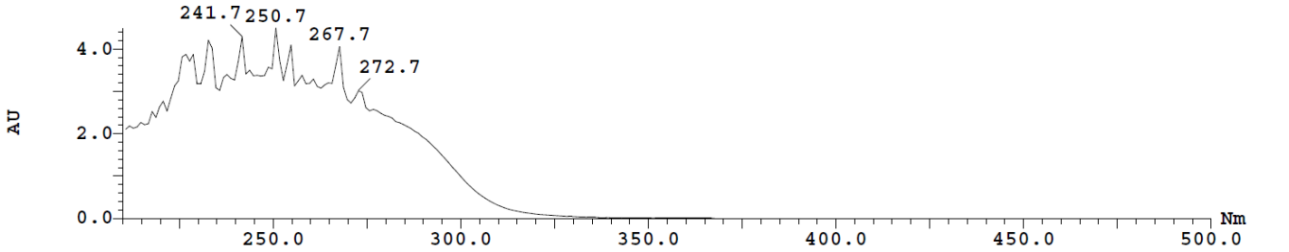
Peak ID	Compound	Time	Mass Found
2		3.38	

2:(Time: 3.38)



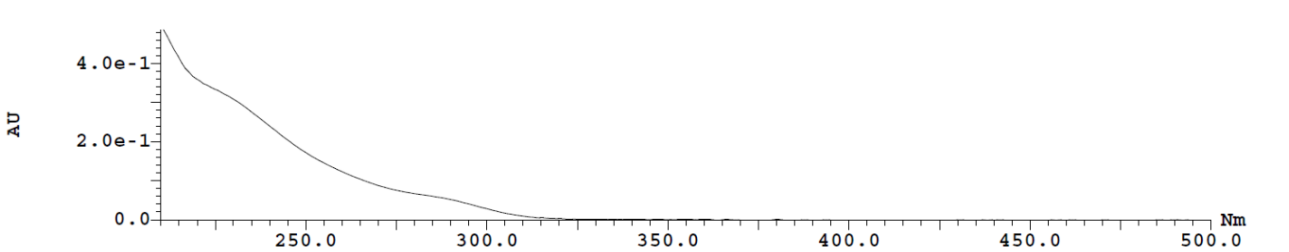
Peak ID	Compound	Time	Mass Found
1		2.29	

1:(Time: 2.30)



Peak ID	Compound	Time	Mass Found
2		3.38	

2:(Time: 3.38)

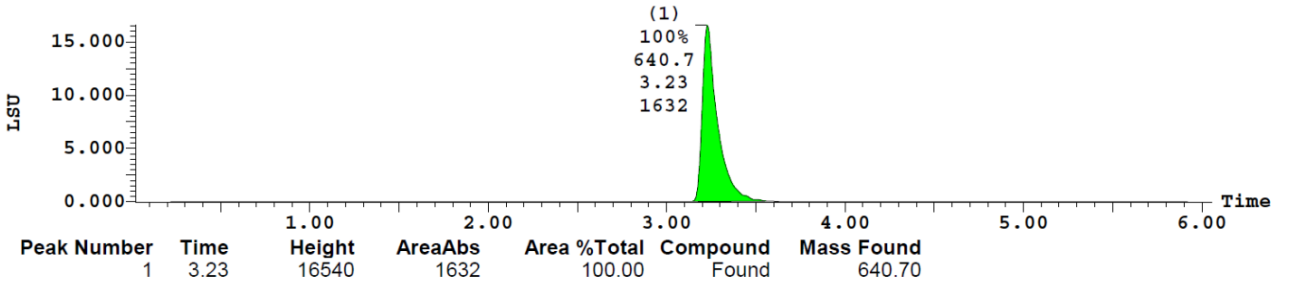


ACE Experimental Record

Sample name:ZC-281-315-3 Probe 3 Vial:5,3:5,E Date:09-Mar-2018
Sample Number: 10 Description:1112 Time:11:50:24

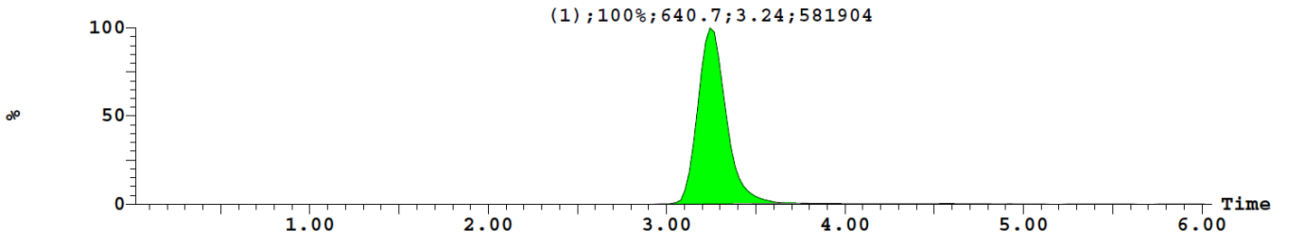
Sample: 10 File: ZC-281-315-3 Column: Kinetex 50 x 4.6

ELSD Signal 16.553
Range: 16.553

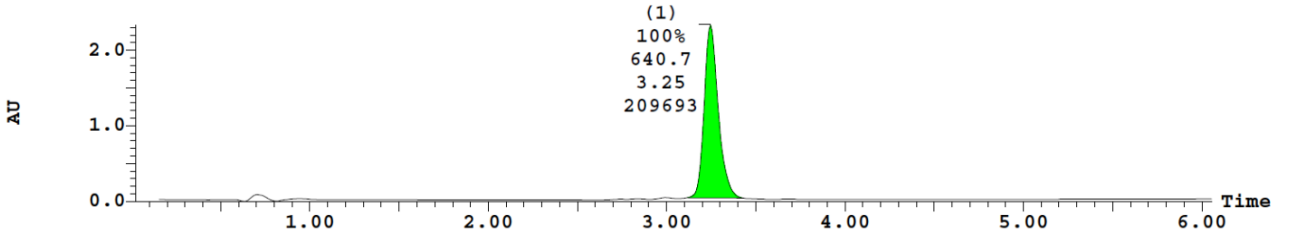


Peak Number	Time	Height	AreaAbs	Area %Total	Compound	Mass Found
1	3.23	16540	1632	100.00	Found	640.70

1: MS ES+ :663.7+641.7 Smooth (Mn, 2x2) 3.2e+006



2: UV Detector: 254 Smooth (Mn, 2x2) 2.332
Range: 2.331



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1	Found	3.25	209693	100.00	0	2287306	640.70

Peak ID	Compound	Time	Mass Found
1	Found	3.24	640.70

1:(Time: 3.23) 1:MS ES+
4.1e+006