Enzyme Mediated Incorporation of Zirconium-89 or Copper-64 into a Fragment Antibody for Same Day Imaging of Epidermal Growth Factor Receptor

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Supplementary Information

Contents

1. Chemical synthesis of H_3L^1 and L^2

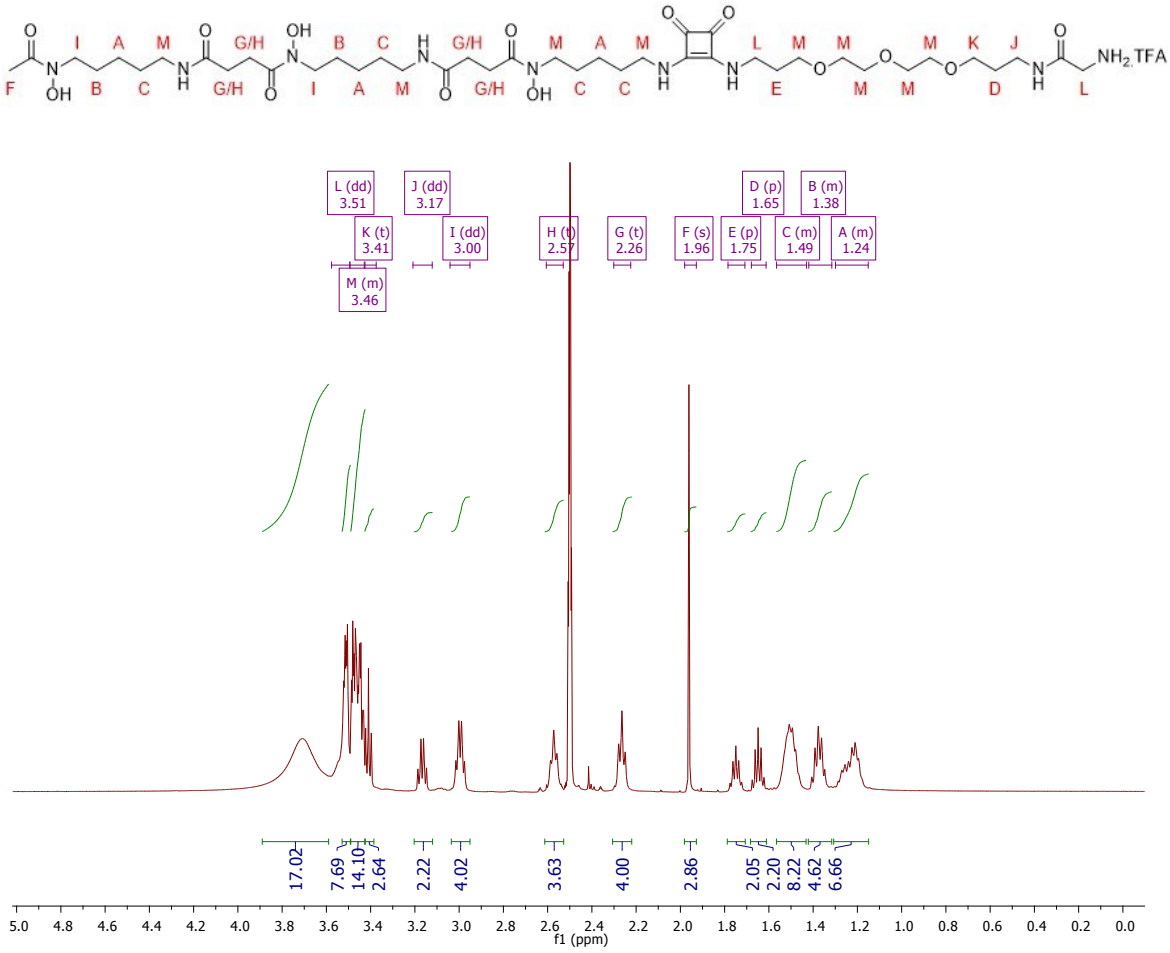
H_3DFOSqOEt. H_3DFOSqOEt was synthesised as previously reported, and characterisation was consistent with that reported.[1]

H_3L^1. A solution of 4,7,10-trioxa-1,13-tridecanediamine (5.563 g, 25.25 mmol) and DIPEA (2.2 mL, 13 mmol) in CH_2Cl_2 (20 mL), and a solution of di-tert-butyl decarbonate (2.78 g, 12.7 mmol) in CH_2Cl_2 (60 mL) was added dropwise. The resulting yellow solution was stirred at ambient temperature for 24 h, then the solvent was removed under reduced pressure. The crude product was purified by silica column chromatography (0-20% MeOH in CH_2Cl_2 with 1% aqueous ammonia) to

give compound **1** as a yellow oil (4.62 g, 57%). ^{1}H NMR (400 MHz, CDCl3) δ 5.16 (s, 1H), 3.64 – 3.43 (m, 12H), 3.21 – 3.10 (m, 2H), 2.76 (t, J = 6.7 Hz, 2H), 1.76 – 1.61 (m, 4H), 1.38 (s, 9H). 321.3009. MS(ESITOF) (m/z): calculated $[C_{15}H_{33}N_2O_5]^+$: 321.24, found: 321.24. Fe^{III}DFOSqOEt (556 mg, 0.754 mmol) was stirred at ambient temperature under N_2 in DMF (10 mL, anhydrous). DIPEA (0.15 mL, 0.86 mmol) was then added, followed by $BocOEG_3NH_2$ (480 mg, 1.50 mmol). The reaction mixture was heated to 45 °C for 48 h, then concentrated to approx. 5 mL under reduced pressure. Water (50 mL) was added and the product extracted into $CHCl_3$ (120 mL) then washed with brine (30 mL). The solvent was removed under reduced pressure to give a mixture of $BocOEG_3NH_2$ and $FeDFOSqOEG_3NHBoc$ as a red oil (916 mg), which was used without further purification. Approximately half of the crude material was stirred at ambient temperature in 15% TFA in CH_2Cl_2 for 2 h, then the volatiles were removed by N_2 flow to give 397 mg of crude product as a red oil. The crude material was then stirred under anhydrous conditions in DMF (20 mL) and DIPEA (1.6 mL, 9.2 mmol), followed by the addition of BocGlyOSu (262 mg, 0.962 mmol). The reaction mixture was stirred at ambient temperature overnight, then the solvent removed under reduced pressure. The red oil was redissolved in H_2O (20 mL) and extracted into $CHCl_3$ (3 x 25 mL). The organic layer was washed with brine, and the solvent removed under reduced pressure to give 288 mg of a red oil which was used in the next step without further purification. The red oil was stirred in a solution of TFA in CH_2Cl_2 (15%, 15 mL) at ambient temperature for 2 h, then the volatiles were removed by N_2 flow. The resulting red oil was then taken up into H_2O (5.5 mL) and heated to 50 °C. A warm solution of aqueous Na_2EDTA (135 mM) was added dropwise until the red colour disappeared (approx. 2 mL in total), leaving a yellow/orange solution. Upon cooling to room temperature, excess Na_2EDTA formed a white precipitate and was filtered off. The yellow filtrate was concentrated under reduced pressure, then purified by semi-preparative HPLC (Supelco Discovery C18 5µm 250 x 10 mm column, 5-20% B over 15 min, then 20% B for 5 min. Retention time 19.5 min. A:0.05% TFA in H_2O, B: 0.05% TFA in MeCN) to give H_3L^1.TFA as a white powder (16 mg, 5% over 5 steps). ^{1}H NMR (500 MHz, d_6-DMSO. Alphabetical position labels are shown in Supp. Fig1.) δ 3.71 (br s, H_2O) 3.51 (dd, J = 5.5, 2.8 Hz, 4H, L), 3.49 – 3.43 (m, 18H, M), 3.41 (t, J = 6.3 Hz, 2H, K), 3.17 (dd, J = 12.7, 6.8 Hz, 2H, J), 3.00 (dd, J = 12.8, 6.7 Hz, 4H, I), 2.57 (t, J = 7.1 Hz, 4H, H), 2.26 (t, J = 7.3 Hz, 4H, G), 1.96 (s, 3H, F), 1.75 (p, J = 6.5 Hz, 2H, E), 1.65 (p, J = 6.6 Hz, 2H, D), 1.57 – 1.43 (m, 8H, C), 1.42 – 1.32 (m, 4H, B), 1.30 – 1.15 (m, 6H, A). ^{13}C NMR (126 MHz, d_6-DMSO) δ 182.35 (squaramide), 182.24 (squaramide), 171.97 (DFO CH_2CONH_2), 171.30 (DFO CH_2CONOH), 170.12 (DFO CH_3CONOH), 167.77 (squaramide), 165.69 (Gly $NHCOCH_2$), 158.45-157.6 (quartet, TFA), 69.76 (L), 69.54 (M), 67.77 (K), 67.41 (M), 47.07 (M), 47.02 (M), 46.78 (M), 43.22 (M), 40.65 (L), 38.42 (I), 35.96 (J),

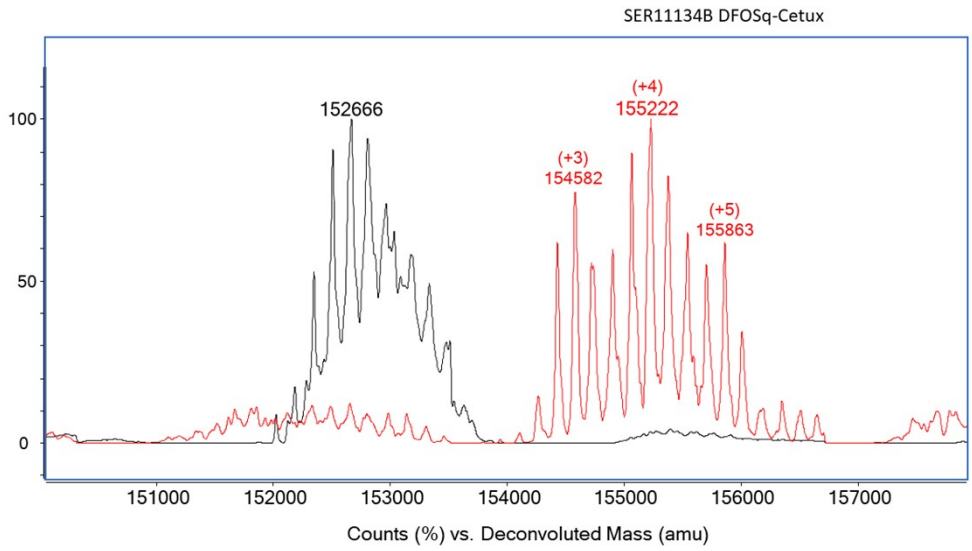
30.85 (E), 30.48 (C), 29.90 (G), 29.11 (D), 28.81 (B), 27.56 (H), 26.03 (C), 23.50 (A), 23.00 (A), 20.35 (F). ESI MS (m/z): $[M+H]^+$ calculated $[C_{41}H_{74}N_9O_{14}]^+$: 916.54, found: 916.53.

L^2. MeCOSarNHS was prepared according to published procedures. and characterisation was consistent with that reported.[2] **L^2** was synthesised according to published procedures, and characterisation was consistent with that reported.[3]



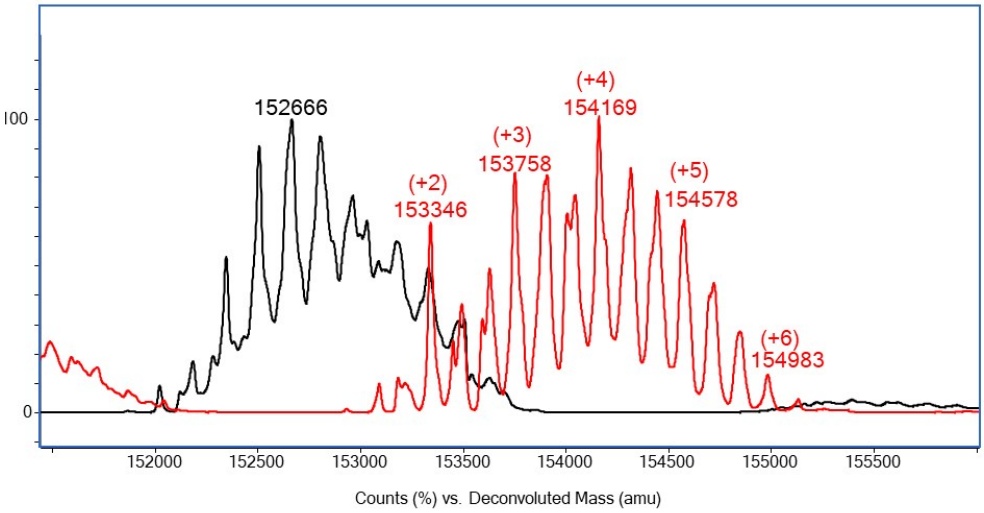
Supplementary Figure 1. Position labels for the assignment of ^{1}H NMR (500 MHz, d_6-DMSO) characterisation data for H_3L^1.

H_3DFOSq-cetuximab



Supplementary Figure 2. Deconvoluted ESI MS of (black) cetuximab prior to conjugation and (red) H_3DFOSq-cetuximab. The number of additions of H_3DFOSq for selected peaks are shown in brackets. All protein samples were analysed using an Agilent 6220 ESI-TOF LC/MS mass spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA).

MeCOSar-cetuximab

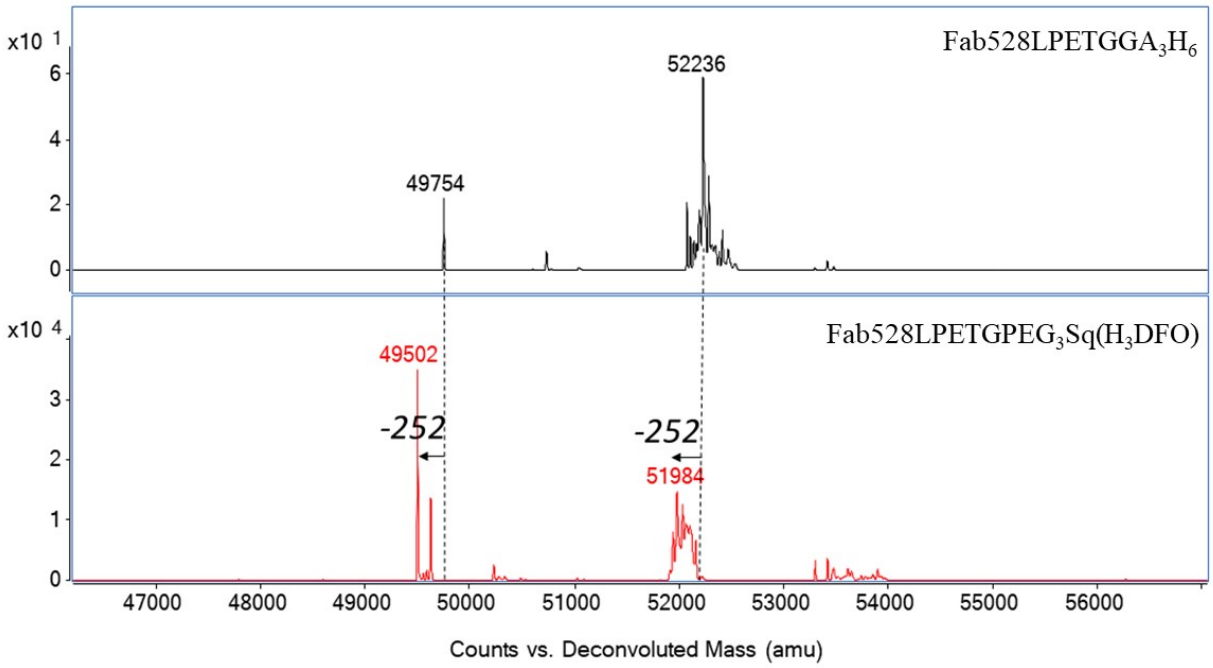


Supplementary Figure 3. Deconvoluted ESI MS of (black) cetuximab prior to conjugation and (red) MeCOSar-cetuximab. The number of additions of MeCOSar for selected peaks are shown in brackets. All protein samples were analysed using an Agilent 6220 ESI-TOF LC/MS mass spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA).

*MKLPVRLLVLMFWIPASSS*DVLMTQTPLSLPVSLGDQASISCRSSQNIVHNNGITYLEWYLQR
PGQSPKLLIYKVSDRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGIYYCFQGSHIPPTFGGGT
KLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWT
DQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC<u>RRKRGSGEGRG
SLLTCGDVEENPGP</u>*MGWSCIILFLVATATGAHS*QVQLQQSGSEMARPGASVKLPCKASGDTF
TSYWMHWVKQRHGHGPEWIGNIYPGSGGTNYAEKFKNKVTLTVDRSSRTVYMHLSRLTS
EDSAVYYCTRSGGPYFFDYWGQGTSLTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVK
GYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTK
VDKKIGGGGSGGGGS**LPETGG**AAADYKDDDDK

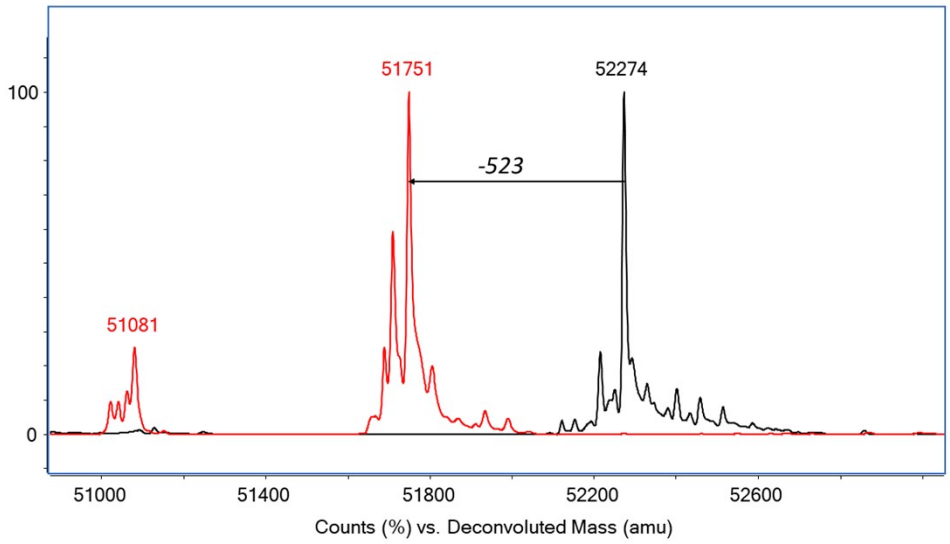
Supplementary Figure 4. Amino acid sequence of Fab528-LPETG-FLAG. Signal peptides for the LC and HC are italicised, while the furin cleavage site (RRKR) and foot-and-mouth disease virus (FMDV) 2A peptide are underlined. The SrtA recognition site at the C-terminus of the HC is in bold text. The signal peptides and 2A peptide are removed during translation.

H_3L^1-Fab528



Supplementary Figure 5. Deconvoluted ESI MS of (top, black) Fab528-LPETG His-tagged variant prior to conjugation and (bottom, red) H_3L^1-Fab528. The mass loss of 252 amu corresponds to the loss of the cleavage peptide plus the addition of L^1. All protein samples were analysed using an Agilent 6220 ESI-TOF LC/MS mass spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA).

L^2-Fab528



Supplementary Figure 6. Deconvoluted ESI MS of (black) Fab528-LPETG FLAG variant prior to conjugation and (red) L^2-Fab528. The mass loss of 523 amu corresponds to the loss of the cleavage peptide plus the addition of L^2. The mass at 51081 corresponds to the hydrolysis product, where the cleavage peptide was removed with the addition of water instead of L^2. All protein samples were analysed using an Agilent 6220 ESI-TOF LC/MS mass spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA).

References

1. Rudd, S. E.; Roselt, P.; Cullinane, C.; Hicks, R. J.; Donnelly, P. S. A desferrioxamine B squaramide ester for the incorporation of zirconium-89 into antibodies. *Chem. Commun.* 2016, 52, 11889-11892.
2. Alt, K.; Paterson, B. M.; Ardipradja, K.; Schieber, C.; Buncic, G.; Lim, B.; Poniger, S. S.; Jakoby, B.; Wang, X.; O'Keefe, G. J.; Tochon-Danguy, H. J.; Scott, A. M.; Ackermann, U.; Peter, K.; Donnelly, P. S.; Hagemeyer, C. E. Single-Chain Antibody Conjugated to a Cage Amine Chelator and Labeled with Positron-Emitting Copper-64 for Diagnostic Imaging of Activated Platelets. *Mol. Pharm.* 2014, 11, 2855-2863.
3. Paterson, B. M.; Alt, K.; Jeffery, C. M.; Price, R. I.; Jagdale, S.; Rigby, S.; Williams, C. C.; Peter, K.; Hagemeyer, C. E.; Donnelly, P. S. Enzyme-Mediated Site-Specific Bioconjugation of Metal Complexes to Proteins: Sortase-Mediated Coupling of Copper-64 to a Single-Chain Antibody. *Angew. Chem. Int. Ed.* 2014, 53, 6115-6119.