Supporting Information:

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4. Trastuzumab IgG and trastuzumab Fab-Alkyne binding positively to other relevant human gastrointestinal tract adenocarcinomas.
5. NIRdye and Ce6; chemical structure and fluorescence spectra.

References:

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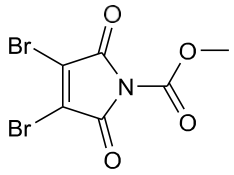
1: Methods for the chemical synthesis of the bridging agent (*N*-propargyl-3,4-dibromomaleimide)

Also see; Castañeda *et al.* 2013 [1]

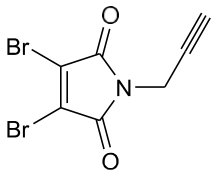
All reactions were carried out at atmospheric pressure with stirring at room temperature (20 °C) unless otherwise stated. All reagents were purchased from Sigma-Aldrich or Thermo Fisher Scientific and were used as received without further purification. All reactions were monitored by thin-layer chromatography (TLC) on pre-coated SIL G/UV254 silica gel plates (254 μm) purchased from VWR. ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker Avance 600 instrument operating at a frequency of 600 MHz for ^{1}H and 150 MHz for ^{13}C in $CDCl_3$. The chemical shifts (δ) for ^{1}H and ^{13}C are quoted relative to residual signals of the solvent on the ppm scale. ^{1}H NMR peaks are reported as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quintet), broad (br) or multiplet (m). Coupling constants (J values) are reported in Hertz (Hz) and are H-H coupling constants unless otherwise stated. Signal multiplicities in ^{13}C NMR were determined using the distortionless enhancement by phase transfer (DEPT) spectral editing technique. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR Spectrometer operating in ATR mode with frequencies given in reciprocal centimetres (cm^{-1}). Melting points were measured with a Gallenkamp apparatus and are uncorrected. Mass spectra were obtained on a VG70-SE mass spectrometer.

3,4-Dibromo-2,5-dioxo-2,5-dihydro-pyrrole-1-carboxylic acid methyl ester



To a solution of 3,4-dibromo-furan-2,5-dione (1.0 g, 3.9 mmol) and *N*-methylmorpholine (0.43 mL, 3.9 mmol) in THF (35 mL) was added methyl chloroformate (0.30 mL, 3.9 mmol) and the reaction mixture stirred at room temperature for 20 min. After this time, CH_2Cl_2 (40 mL) was added, the organic phase washed with H_2O (3 × 40 mL), dried ($MgSO_4$) and the solvent removed *in vacuo* to afford 3,4-dibromo-2,5-dioxo-2,5-dihydro-pyrrole-1-carboxylic acid methyl ester as a pink powder (1.18 g, 3.8 mmol, 97%): m.p. 114-116 °C (*lit. m.p. 115-118 °C*)[1]; ^{1}H NMR ($CDCl_3$, 600 MHz) $\underline{\delta}_H$: 4.00 (3H, s, C*H*$_3$); ^{13}C NMR ($CDCl_3$, 150 MHz) $\underline{\delta}_C$: 159.3 (C), 147.0 (C), 131.5 (C), 54.9 (CH_3); IR (solid) 3236, 2962, 1809, 1769, 1730, 1602 cm^{-1}; LRMS (CI) 314 (50, $[M^{81}Br^{81}Br+H]^+$), 312 (100, $[M^{81}Br\ ^{79}Br+H]^+$), 310 (50, $[M^{79}Br\ ^{79}Br+H]^+$); HRMS (EI) calcd for $C_6H_3O_4NBr_2$ $[M^{81}Br^{79}Br]^+$ 310.8423, observed: 310.8427.

***N*-propargyl-3,4-dibromomaleimide**



Propargylamine (0.049 mL, 0.77 mmol) was added to a stirred solution of 3,4-dibromo-2,5-dioxo-2,5-dihydro-pyrrole-1-carboxylic acid methyl ester (200 mg, 0.64 mmol) in dichloromethane (8 mL). After 20 min, EtOAc (20 mL) was added and the organic layer washed with 15% aq. citric acid (20 mL) and water (2×20 mL), dried ($MgSO_4$) and concentrated *in vacuo* to yield a pale yellow solid that was purified by flash chromatography on silica gel in petroleum ether:EtOAc (6:1 v/v) to afford *N*-propargyl-3,4-dibromomaleimide as a white solid (138 mg, 0.47 mmol, 74%): m.p. 118-120 ºC (*lit. m.p. 119-120 °C*)[1]; ^{1}H NMR ($CDCl_3$, 600MHz) $\underline{\delta}_H$: 2.26-2.28 (t, *J* = 2.4 Hz, C*H*), 4.36-4.37 (d, *J* = 2.4 Hz, C*H*$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) $\underline{\delta}_C$: 162.6 (CO), 129.8 (C), 76.1 (C), 72.6 (CH), 28.6 (CH_2); IR (solid) 3293, 3263, 1719, 1588 cm^{-1}; HRMS (EI) calcd. for $C_7H_3NO_2Br_2$ $[M^{79}Br^{79}Br]^+$ 290.85305, observed: 290.85347.

Figure S1: NMR of 3,4-dibromo-2,5-dioxo-2,5-dihydro-pyrrole-1-carboxylic acid methyl ester

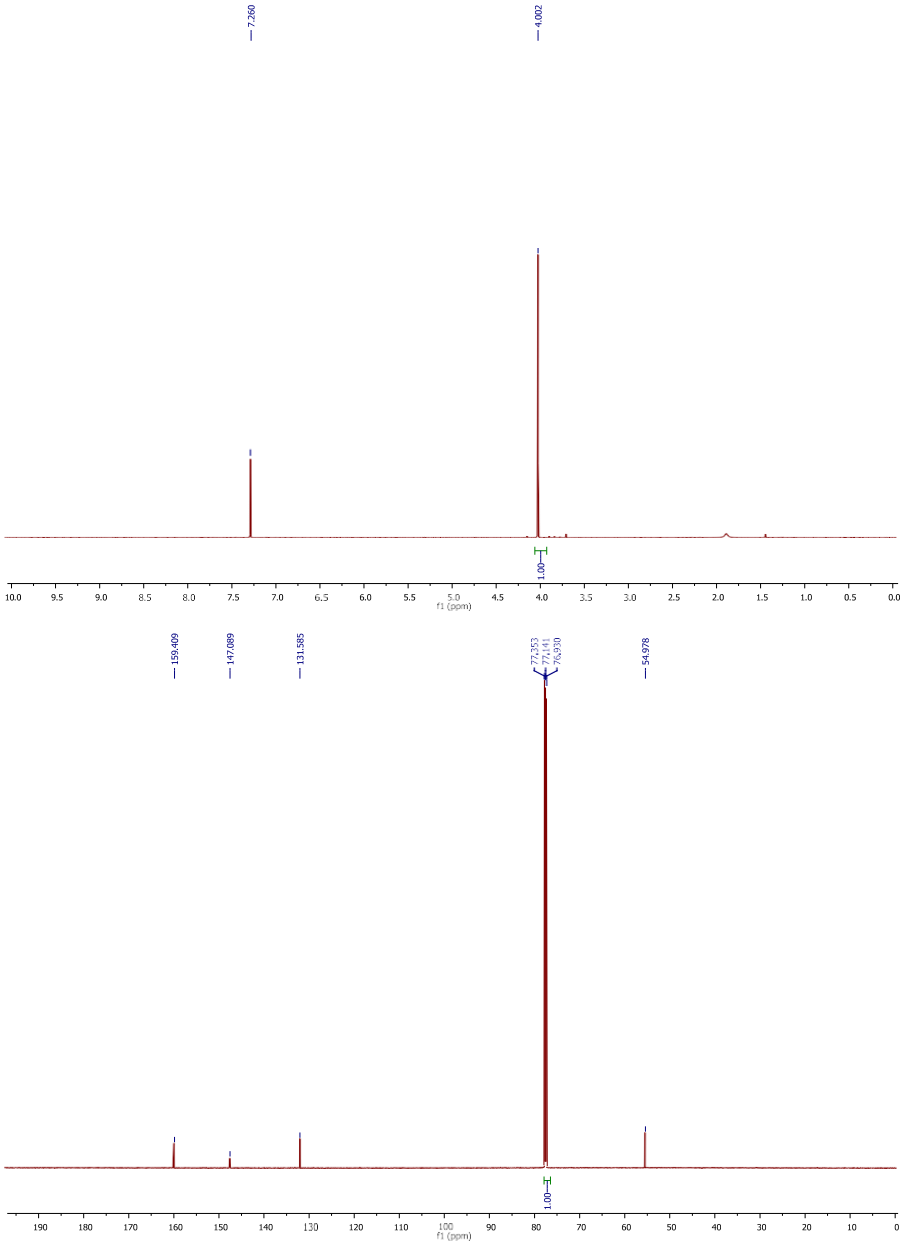
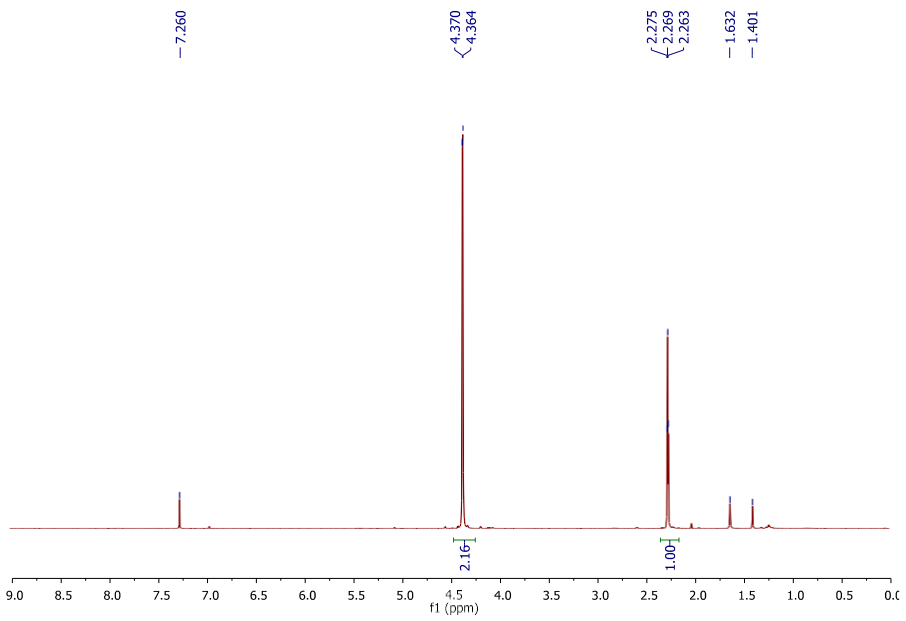
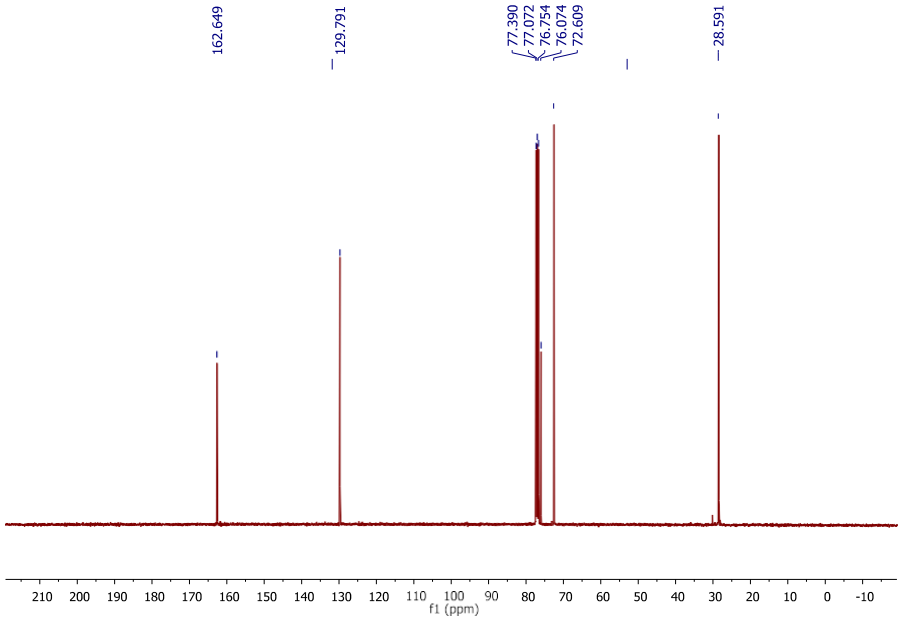


Figure S2: NMR of *N*-propargyl-3,4-dibromomaleimide





2: Detail on production of high purity trastuzumab Fab Alkyne

General remarks:
Conjugation experiments were carried out in standard polypropylene micro test tubes 3810x at atmospheric pressure with mixing at 20 °C, unless otherwise stated. Reagents and solvents were purchased from commercial sources and used as supplied. All buffer solutions were prepared with double-deionised water and filter-sterilised. Digest buffer 1 was 20 mM sodium acetates, pH 3.1. Digest buffer 2 was 150 mM NaCl, 50 mM sodium phosphates, 1 mM EDTA, pH 6.8. Borate buffer was 25 mM sodium borate, 25 mM NaCl and 1 mM EDTA, pH 8.0. Phosphate-buffered saline (PBS) was 140 mM NaCl and 12 mM sodium phosphates at pH 7.4 Ultrapure DMF was purchased from Sigma-Aldrich and kept under dry conditions. Solutions of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 6.5 mM (1.86 mg/mL) were prepared in borate buffer. Filtration of particulates was carried out through Spin-X 0.22 μm cellulose acetate filters. Ultrafiltration was carried out in vivaspin 500 polyethersulfone (PES) membrane concentrators with a molecular weight cut-off (MWCO) of 10 kDa or in Amicon Ultra-15 low binding cellulose filters with 10 kDa MWCO. Centrifugation was carried out on an eppendorf 5415R fixed angle rotor centrifuge operating at 14000 rcf at 20 °C or in an eppendorf 5810 swing-bucket rotor centrifuge operating at 3220 rcf at 20 °C.

Trastuzumab is a chimeric IgG1 full length antibody directed against HER2. The antibody was obtained in its clinical formulation (Roche, lyophilised), dissolved in 10 ml sterile water and the buffer exchanged completely for 20 mM sodium acetates pH 3.1 *via* ultrafiltration (MWCO 10 kDa, Amicon). Concentration was determined by UV-vis absorbance (using ε_{280} = 215380 M^{-1} cm^{-1} for trastuzumab mAb) and adjusted to 44 μM (6.4 mg/mL). Trastuzumab mAb was digested to trastuzumab antigen binding fragment (trastuzumab Fab) as described in the digest protocol section using immobilised pepsin and immobilized papain (cross-linked, 6%, beaded agarose, 50% glycerol slurry in 0.1 M acetate, pH 4.5, 0.05% sodium azide), both purchased from Thermo Scientific.

SDS-PAGE:
Non-reducing glycine-SDS-PAGE 12% acrylamide gels were performed following standard lab procedures. A 4% stacking gel was used and a broad-range MW marker (10–250 kDa, BioLabs) was co-run to estimate protein weights. Samples (3 μL at ~65 μM in total trastuzumab Fab) were quenched with maleimide (1 μL of a 12 mM solution in PBS, >100 eq.) and mixed with loading buffer (2 μL, composition for 6× SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH 6.8, 2 mg R-250 dye) and heated at 65 °C for 2 minutes. For reducing gel (using β-mercaptoethanol (BME) as reducing agent), samples (3 μL at ~65 μM in total trastuzumab Fab) were mixed with loading buffer (2 μL, composition for 4 x SDS: 0.8 mL BME, 0.8 g SDS, 4 mL glycerol, 2.5 mL 0.5 M Tris buffer pH 6.8, 2.5 mL H_2O, 2 mg R-250 dye). The gel was run at constant current (30-35 mA) for 40 min in 1× SDS running buffer. All gels were stained following a modified literature protocol **(Candiano et al 2004)**[2] where 0.12 % of Coomassie G-250 and Coomassie R-250 dyes were added to the staining solution (5:4:1 MeOH:H_2O:AcOH).

Determination of trastuzumab Fab and conjugate concentration:
UV-vis spectra were recorded on a Varian Cary 100 Bio UV-visible spectrophotometer, operating at 20 °C. Sample buffer was used as blank for baseline correction. Calculation of antibody fragment concentration followed the Beer-Lambert law using ε_{280} = 68590 M^{-1} cm^{-1} for trastuzumab Fab. Trastuzumab Fab conjugate concentration was determined using the same extinction coefficient as the maleamic acid group was found to have negligible absorption at 280 nm compared to trastuzumab Fab.

Liquid chromatography mass spectrometry (LC-MS):
Trastuzumab Fab and respective conjugate samples were prepared in water (4.2 μM, 0.2 mg/mL). LC-MS analysis was performed on an Agilent 1100 HPLC connected to a Micromass Q-TOF API-US. Column: Hypersil Gold C4, 1.9 μm 2.1 × 50 mm. Wavelength: 254 nm. Mobile Phase: 95:5 Water:MeCN (0.1% formic acid) to 5:95 Water:MeCN (0.1% formic acid) gradient over 7-20 min. Flow Rate: 0.4 mL/min. MS Mode: ES+. Scan Range: m/z = 500–2400. Scan time: 1.0 s. Data

obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 35 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 650 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the 2.8-3.3 or 4-5 min range. Total mass spectra for protein samples were reconstructed from the ion series using MassLynx V4.0 SP4 software.

Digest of trastuzumab to afford trastuzumab Fab:

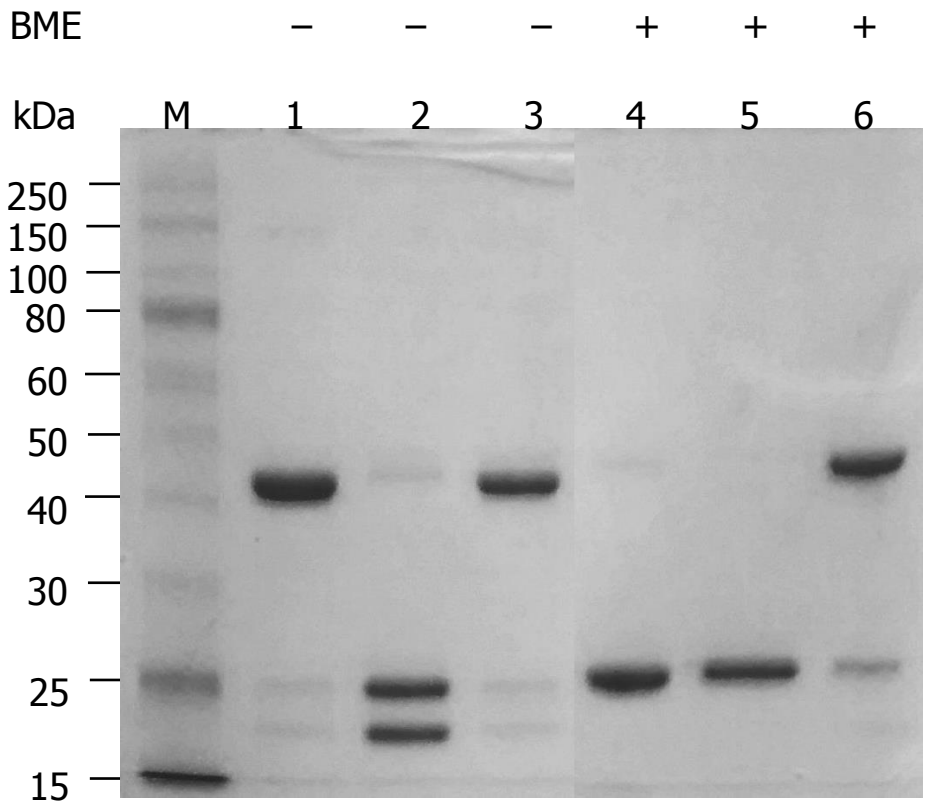
Pepsin digest: Immobilised Pepsin (0.6 mL of Pepsin resin) was placed in a Spin-X 0.22 µm cellulose acetate filter and washed with digest buffer 1 (20 mM acetates, pH 3.1, minimum of 4 washes with 0.4 mL per wash). The immobilised Pepsin was mixed with a solution of trastuzumab mAb (2 mL at 44 µM, 0.087 µmol) in digest buffer 1 and incubated at 37 °C, 1100 rpm, for 5 hours. Then, the trastuzumab Fab_2 solution thus generated was separated from immobilised Pepsin by filtration in a Spin-X 0.22 µm cellulose acetate filter. Immobilised Pepsin was washed further with digest buffer 2 (150 mM NaCl, 50 mM phosphates, 1 mM EDTA, pH 6.8, minimum of 3 washes with 0.4 mL per wash). Collected and combined the filtrate with all washes (Fab_2 solution). Buffer swapped into digest buffer 2 by ultrafiltration (10 kDa MWCO) and corrected the final volume to 2 mL.

Papain digest: Immobilised Papain (2 mL of Papain resin) was placed in a Spin-X 0.22 µm cellulose acetate filter and washed with 15 mM DTT in digest buffer 2 (minimum 4 washes with 0.5 mL per wash volume). Final volume was corrected to 2 mL with 15 mM DTT in digest buffer 2. Immobilised Papain was incubated at 37 °C, 1100 rpm, for 1 hour. Next, washed activated immobilised Papain with digest buffer 2 (no DTT, minimum 6 washes with 0.6 mL wash volume). The trastuzumab Fab_2 solution was mixed with the immobilised Papain and incubated at 37 °C, 1100 rpm, for 48 hours. Then, the trastuzumab Fab solution thus generated was separated from immobilised Papain by filtration in a Spin-X 0.22 µm cellulose acetate filter. Immobilised Papain was washed further with borate buffer (25 mM NaCl, 25 mM sodium borate, 1 mM EDTA, pH 8, minimum of 3 washes with 0.4 mL per wash). Collected and combined the filtrate with all washes (Fab solution). Buffer swapped into borate buffer by ultrafiltration (10 kDa MWCO) and corrected the final volume to 2 mL (6.17 mg, overall yield 74%).

Conjugation of trastuzumab Fab with *N*-propargyl-3,4-dibromomaleimide:

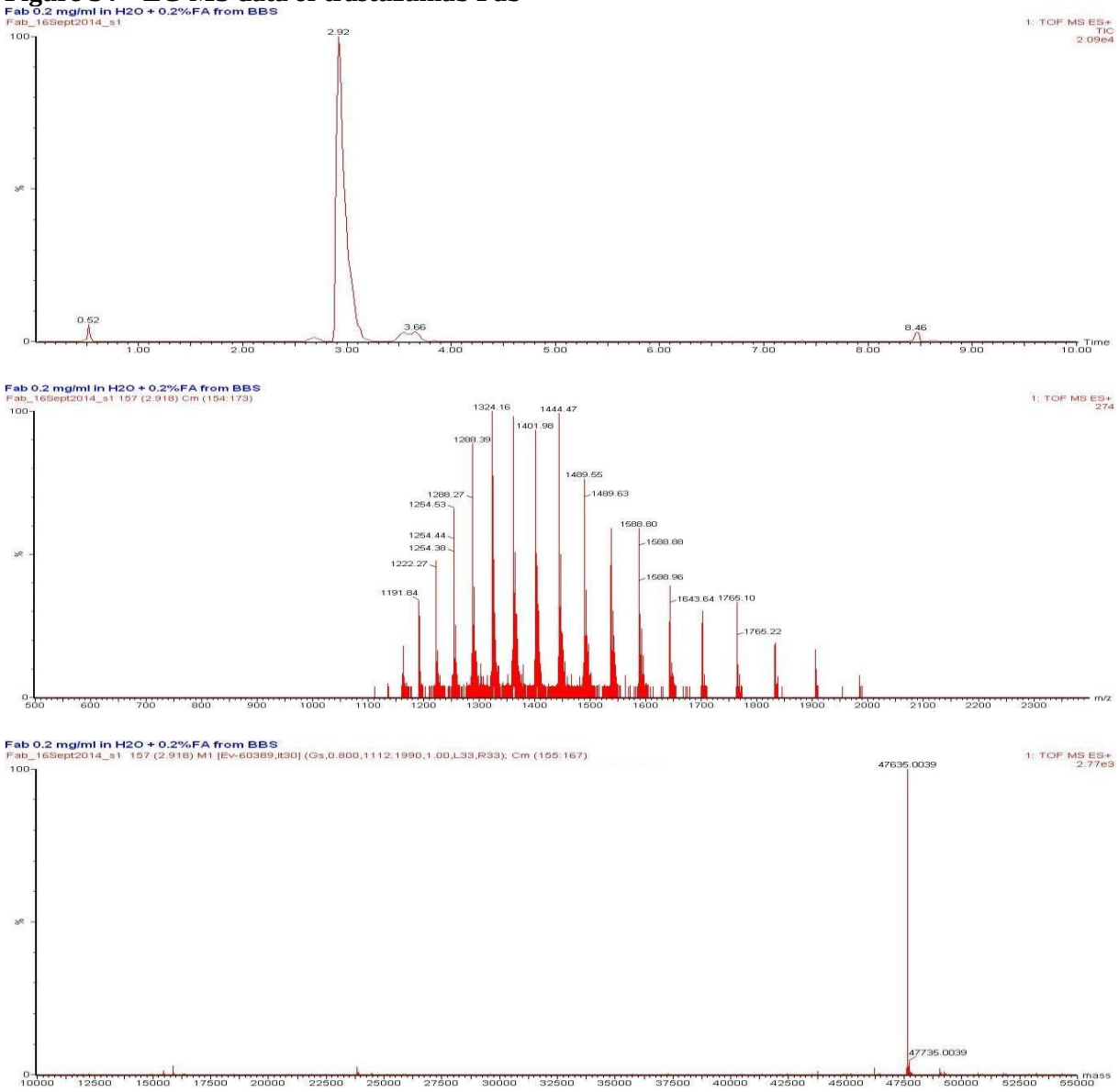
To 8 mL of trastuzumab Fab solution in borate buffer (65 µM, 0.52 µmol, 24.8 mg) was added TCEP in borate buffer (6.5 mM, 240 µL, 3 eq.) and the solution was incubated at 37 °C, 450 rpm, for 1.5 h. Next, added *N*-propargyl-3,4-dibromomaleimide prepared in dry DMF (6.5 mM, 400 µL, 5 eq.) and the solution was incubated at 20 °C, 450 rpm, for 1 h. Afterwards, excess reagents were removed by ultrafiltration (10 kDa MWCO) with PBS to afford the modified trastuzumab Fab conjugate in 8 mL of PBS with 96% yield (62.4 µM, 0.50 µmol, 23.8 mg).

Figure S3 – SDS-PAGE gel of trastuzumab Fab conjugated with *N*-propargyl-3,4-dibromomaleimide:



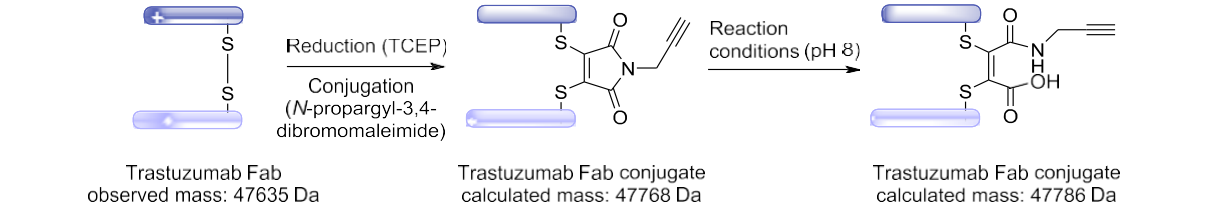
SDS-PAGE gel of trastuzumab Fab conjugated with *N*-propargyl-3,4-dibromomaleimide. M) protein ladder. 1) unmodified trastuzumab Fab (no BME). 2) reduced trastuzumab Fab prior to conjugation (no BME). 3) Trastuzumab Fab conjugated with *N*-propargyl-3,4-dibromomaleimide (no BME). 4) unmodified trastuzumab Fab (with BME). 5) reduced trastuzumab Fab prior to conjugation (with BME). 6) Trastuzumab Fab conjugated with *N*-propargyl-3,4-dibromomaleimide (with BME).

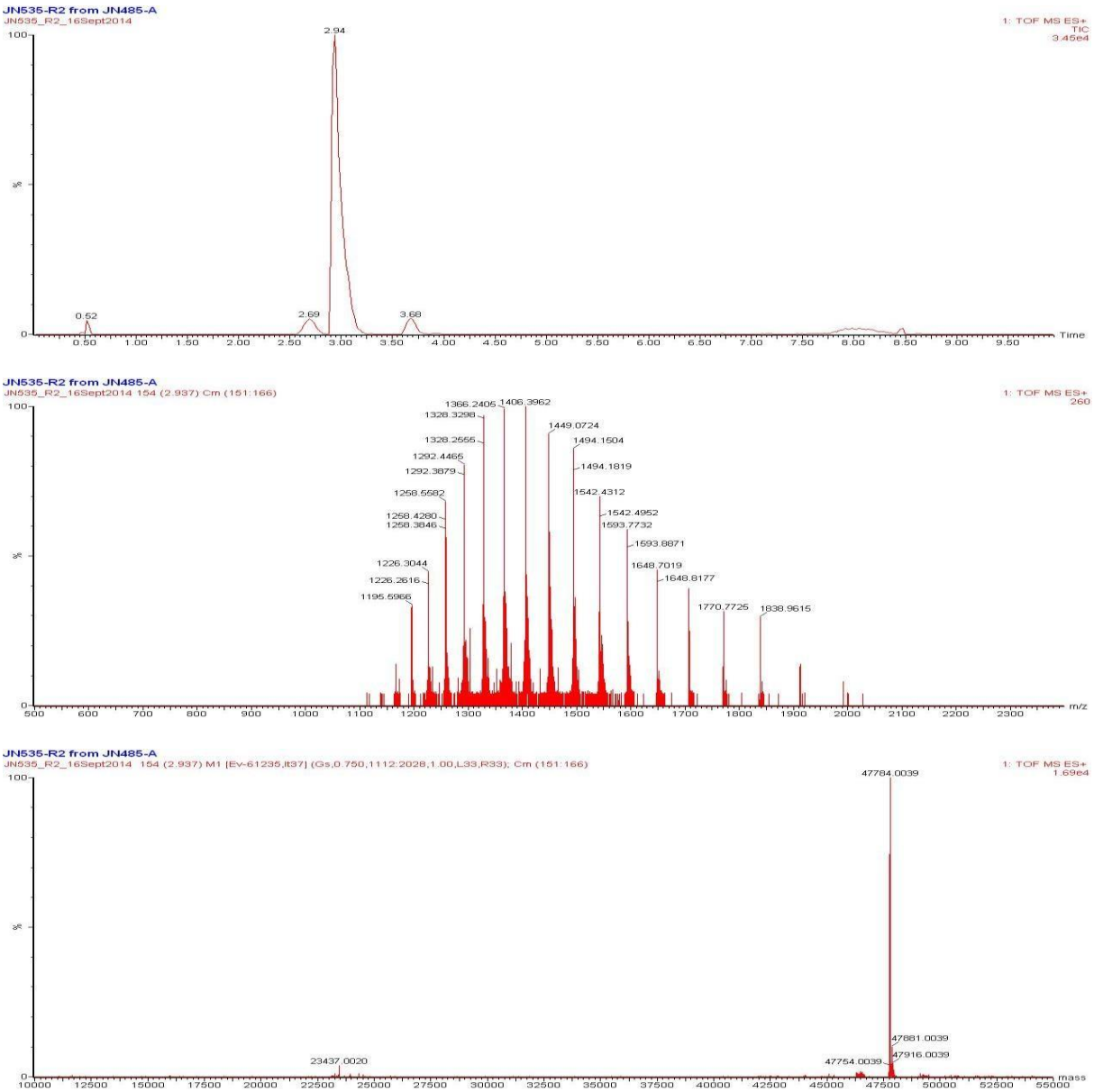
Figure S4 – LC-MS data of trastuzumab Fab



LC-MS data for trastuzumab Fab. **TOP** Total ion count spectra, **MIDDLE** non-deconvoluted and **BOTTOM** deconvoluted mass spectra (observed mass 47635).

Figure S5 – LC-MS data of trastuzumab Fab conjugated with *N*-propargyl-3,4-dibromomaleimide:



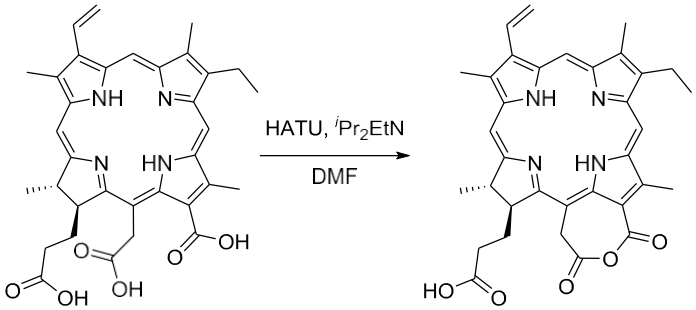


LC-MS data for trastuzumab Fab conjugate with *N*-propargyl-3,4-dibromomaleimide. **TOP** Total ion count spectra, **MIDDLE** non-deconvoluted and **BOTTOM** deconvoluted mass spectra (calculated mass 47786 Da, observed mass 47784 Da).

3: Methods for the chemical synthesis of the anhydride-Ce6

Modified from Chen *et al.* 2015 [3] and Xu et al 2008 [4]

Chlorin e_6 13^1:15^2-anhydride

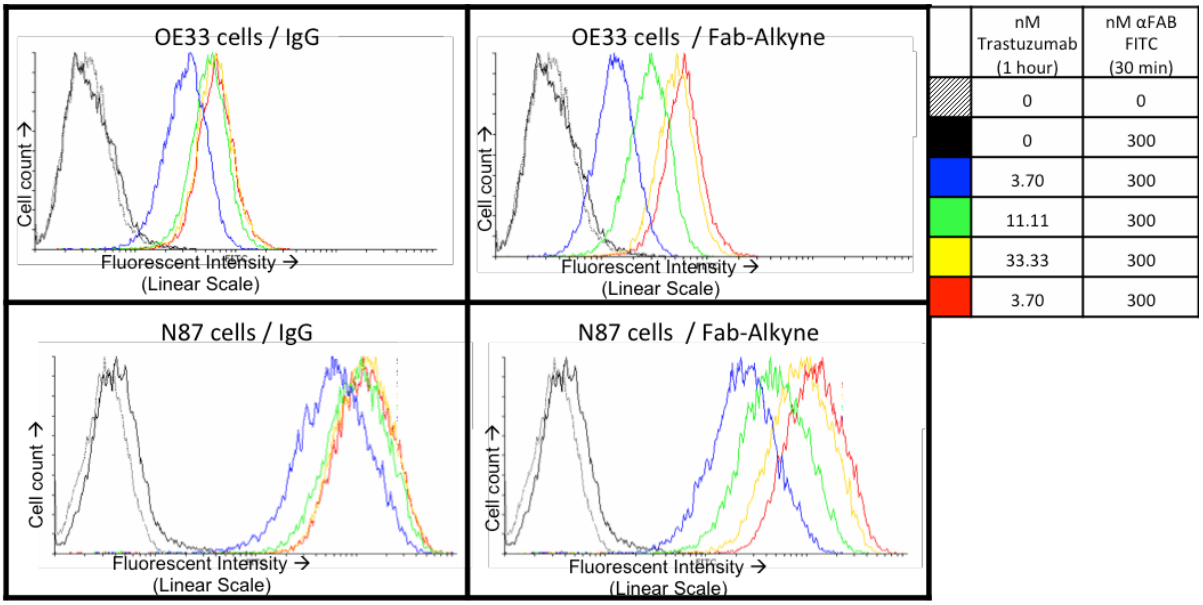


To a stirred solution of chlorin e_6 (100 mg, 0.17 mmol) and *N*,*N*-diisopropylethylamine (58 µL, 0.15 mmol) in anhydrous DMF (2 mL) at room temperature was added HATU (57 mg, 0.15 mmol) and stirred protected from light for 1 h. The crude reaction mixture was then concentrated under reduced pressure and purified by preparative TLC eluting with anhydrous acetone and the residue recrystallised from dichloromethane with *n*-hexane. MS (ES-ToF) *m/z* 579.2 $[M+H]^+$

4: Trastuzumab IgG and trastuzumab Fab-Alkyne binding positively to other relevant human gastrointestinal tract adenocarcinomas

Trastuzumab in its full IgG format has specificity toward the cancer antigen HER2, binding to two upper GI cell lines was tested with the IgG form of Trastuzumab alongside the 'Alkyne-FAB version' of the antibody. The additional cells tested were OE33 an epithelial human cell line established from a barrett's associated stage two poorly differentiated adenocarcinoma of the lower oesophagus **(Rockett *et al.* 1997)**[5] and N87 (NCI-N87) a human epithelial cell line established from a well differentiated gastric carcinoma **(Park *et al.* 1990)**[6]. An increase in fluorescence represents more trastuzumab has bound to each cell and the saturation of the fluorescent signal indicates cell surface receptor saturation. Very little binding affinity is lost in the Fab despite the loss of bi-valency.

Figure S6 – Trastuzumab IgG and Fab-Alkyne binding to OE33 and N87 cells.



The *in vitro* adherent cell lines were detached with Accutase, and Approx. 200,000 cells per sample were washed and incubated in 50 µL on ice with varying concentrations of Trastuzumab. After 1 hour cells were again washed and exposed to 300 nM α-Human IgG (Fab specific) FITC conjugate (SIGMA F5512) on ice for 30 minutes before two final washes. All steps carried out in FACS buffer; PBS + 2% FCS + 1 mM EDTA. Flow cytometry was carried out on a Beckman-Coulter Cyan ADP, Cells underwent laser excitation at 488 nm and emission was recorded between 510 nm and 550 nm.

5: NIRdye and Ce6; chemical structure and fluorescence spectra.

NIR dye was obtained commercially from Licor (IRDye 800CW Azide Infrared Dye), Chlorin-e6 (Ce6) was obtained commercially from (Medkoo Biosciences Cat. 500410), For fluorescent spectral analysis samples were diluted in PBS pH7.4 into a small volume quartz cuvette to ensure peak absorbance was below 0.25 absorbance units (Agilent 8453 spectrophotometer). The cuvette was transferred to the spectrofluorometer (Horiba Jobin Yvon Fluoromax-4) and maintained at 20-22 °C. The emission spectra was corrected at each wavelength for the sensitivity of the detector and the excitation spectra was corrected at each wavelength for the lamp intensity (FluorEssence). Any wavelength over the second harmonic were disreguarded from the presented excitation spectra. Spectra were normalised to the maximal counts per second (CPS) in each sample. Chemical Structures were drawn in MarvinSketch Version 15.8.31.0 (ChemAxon Ltd.) and based on supplier's information.

Figure S7 – chemical structure and fluorescence spectra of Ce6 and NIRdye