Supplementary Information

Homogeneous Bispecifics by Disulfide Bridging

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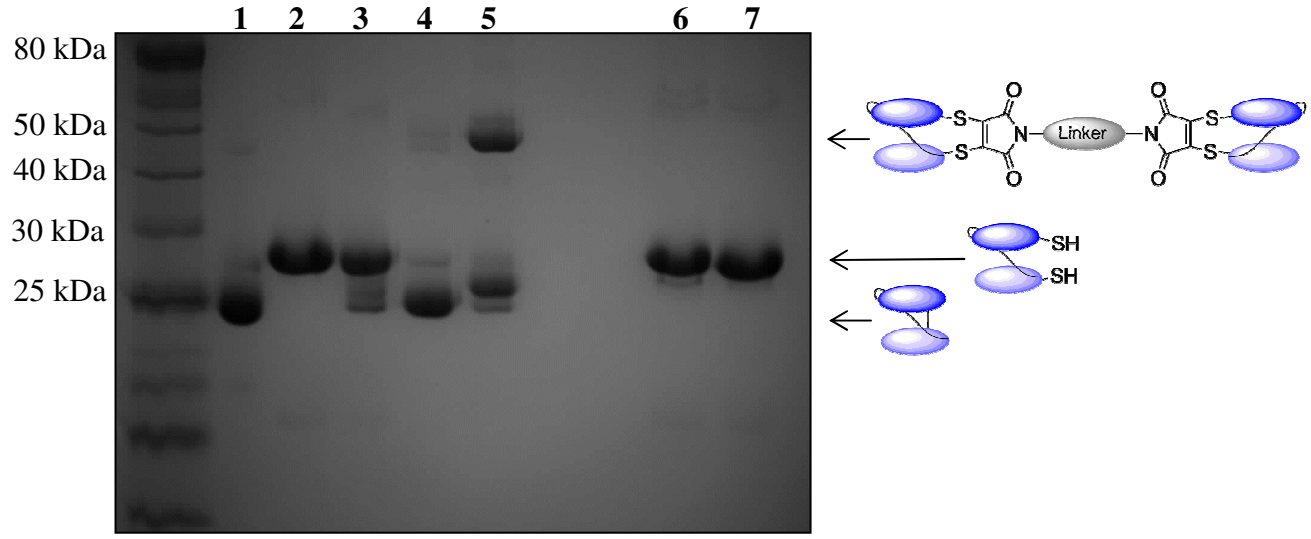
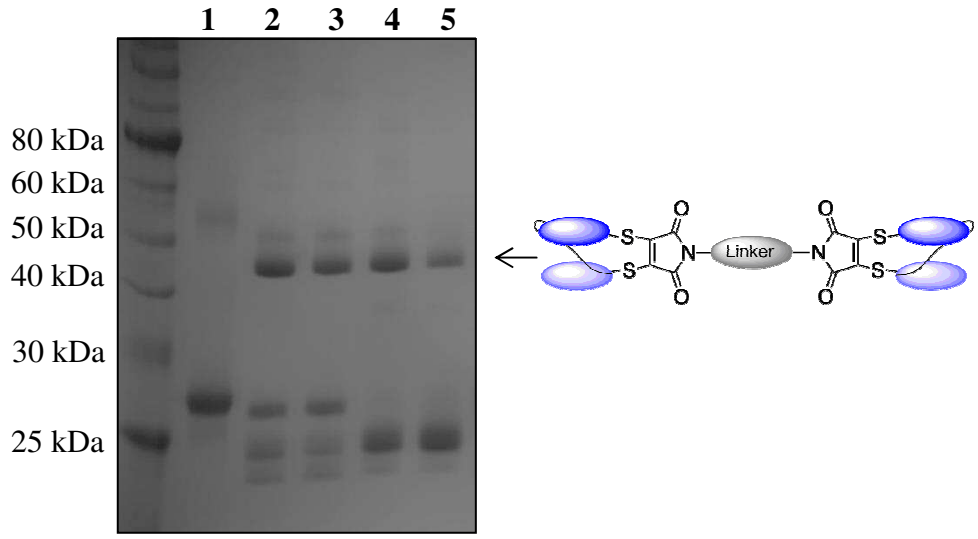
Supplementary Figure 1 – SDS-PAGE analysis: scFv with BDBM(PEG)$_2$ and BDBM(PEG)$_{19}$.



Initial investigation into the formation of scFv homodimer with linkers **BDBM(PEG)$_2$** and **BDBM(PEG)$_{19}$**. *Lane 1*; unmodified scFv. *Lane 2*; scFv + DTT to afford reduced scFv (scFv with intact disulfide runs faster through gel due to more compact structure). *Lane 3*; Reduced scFv (after buffer exchange) left for duration of experiment. *Lane 4*; Reduced scFv + **BDBM(PEG)$_2$** to afford mainly disulfide bridged scFv (~25 kDa). *Lane 5*; Reduced scFv + **BDBM(PEG)$_{19}$** to afford scFv homodimer (~50 kDa) and disulfide bridged scFv monomer (~25 kDa). *Lane 6*; sample from lane 4 + reducing loading buffer. *Lane 7*; sample from lane 5 + reducing loading buffer.

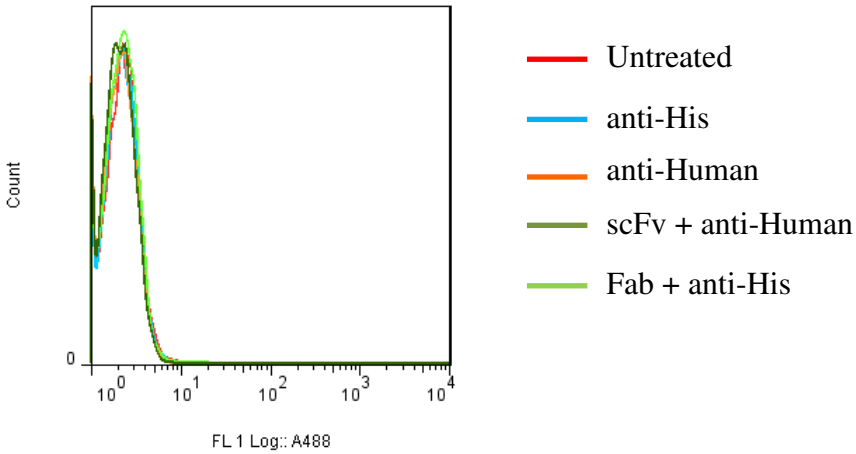
Supplementary Figure 2 – SDS-PAGE analysis: Optimization of homodimer yield using varying equivalents of BDBM(PEG)$_{19}$.



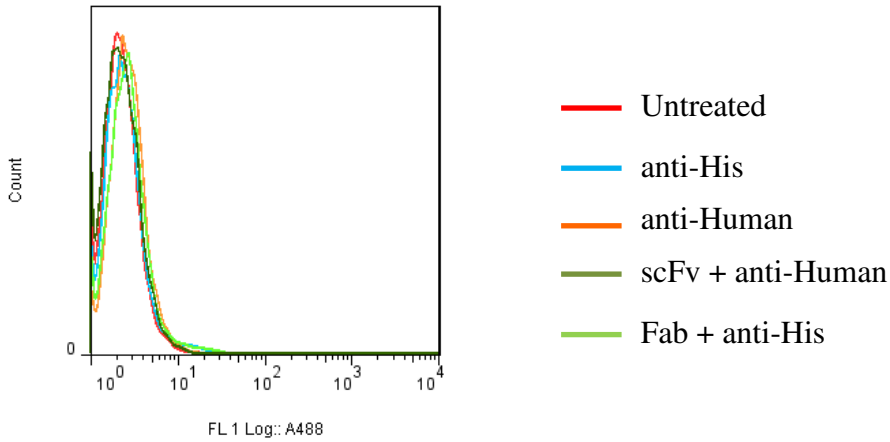
Optimizing yield of scFv homodimer by varying equivalents of linker **BDBM(PEG)$_{19}$**. *Lane 1*; Reduced scFv. *Lane 2*; scFv + 0.42 equiv. **BDBM(PEG)$_{19}$**. *Lane 3*; scFv + 0.5 equiv. **BDBM(PEG)$_{19}$**. *Lane 4*; scFv + 1.0 equiv. **BDBM(PEG)$_{19}$**. *Lane 5*; scFv + 2.0 equiv. **BDBM(PEG)$_{19}$**.

Supplementary Figure 3 – FACs controls

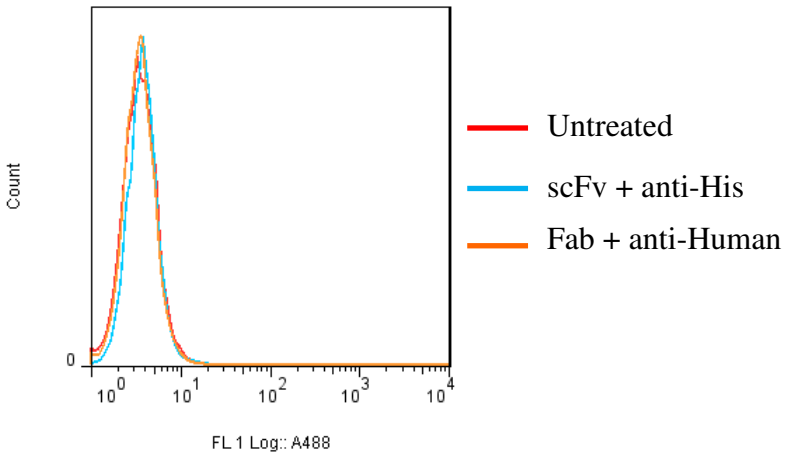
CEA-positive cell line A375CEA controls

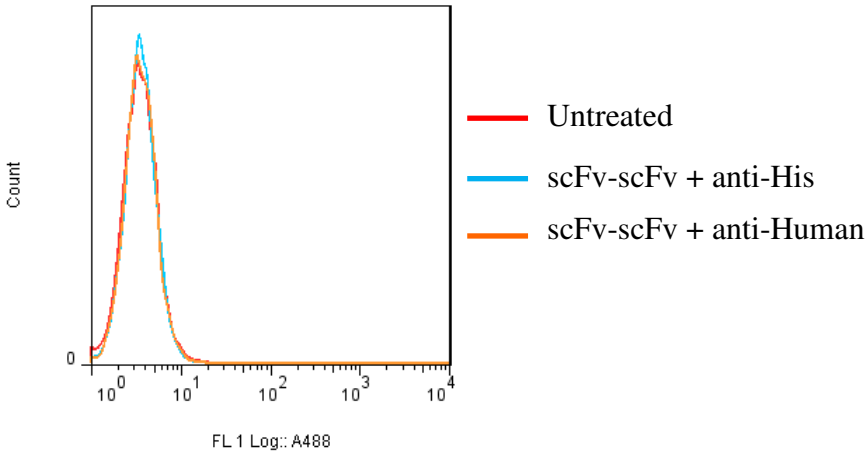


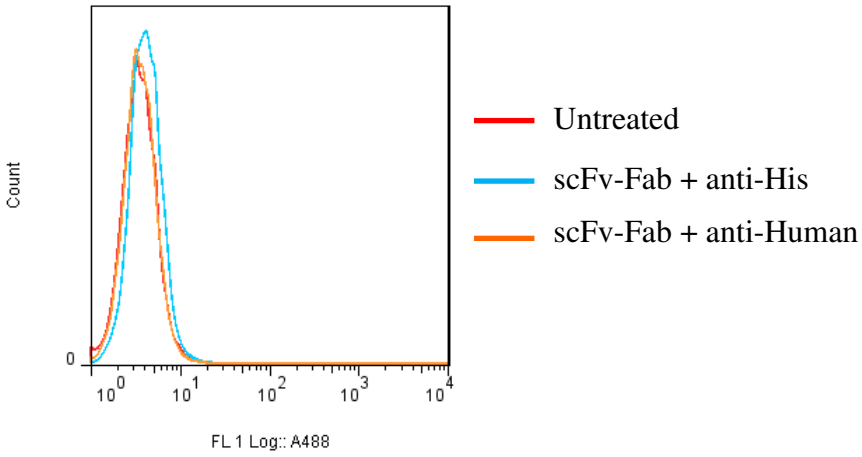
HER2-positive cell line BT474 controls

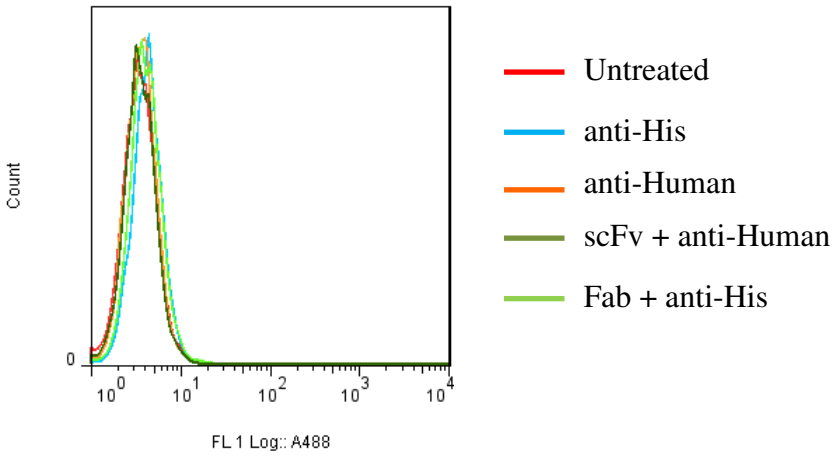


CEA-negative and HER2-negative cell line MDA-MD-468



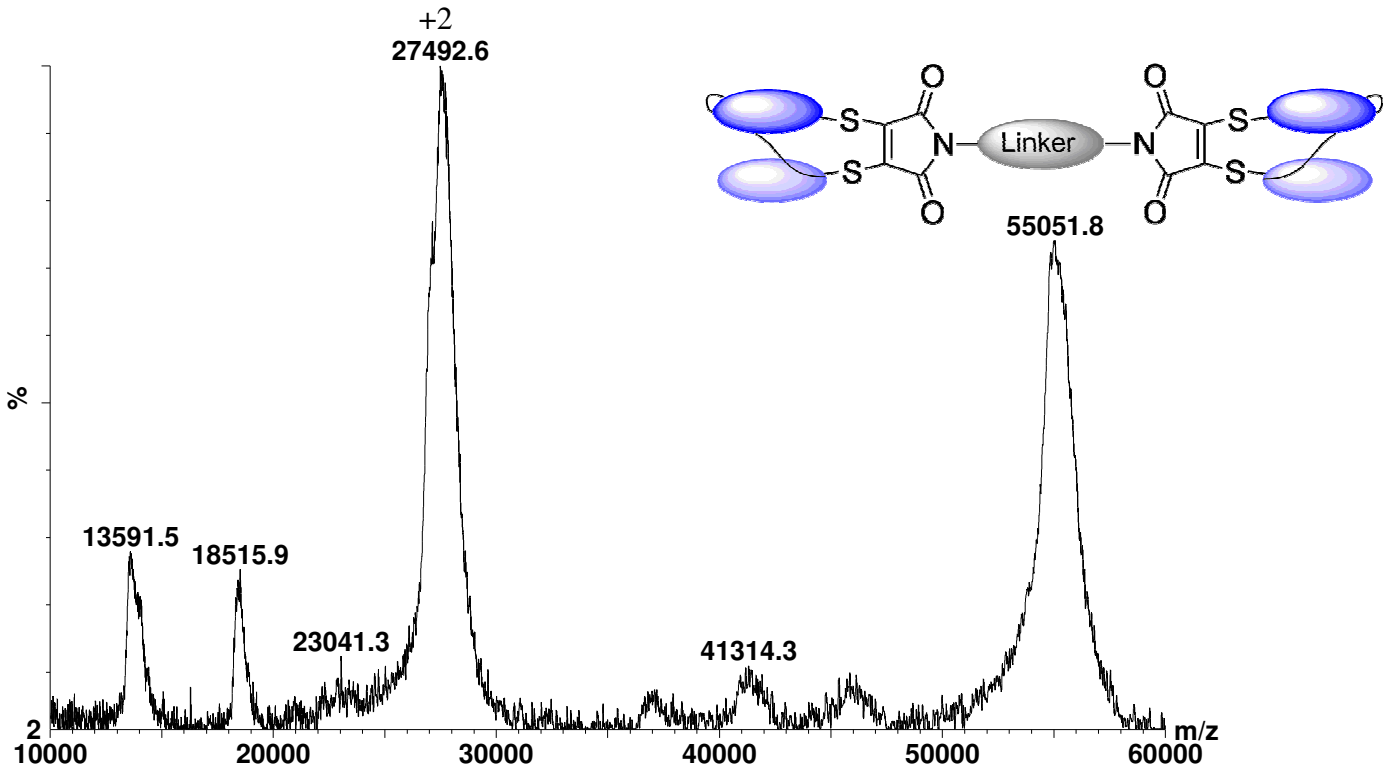


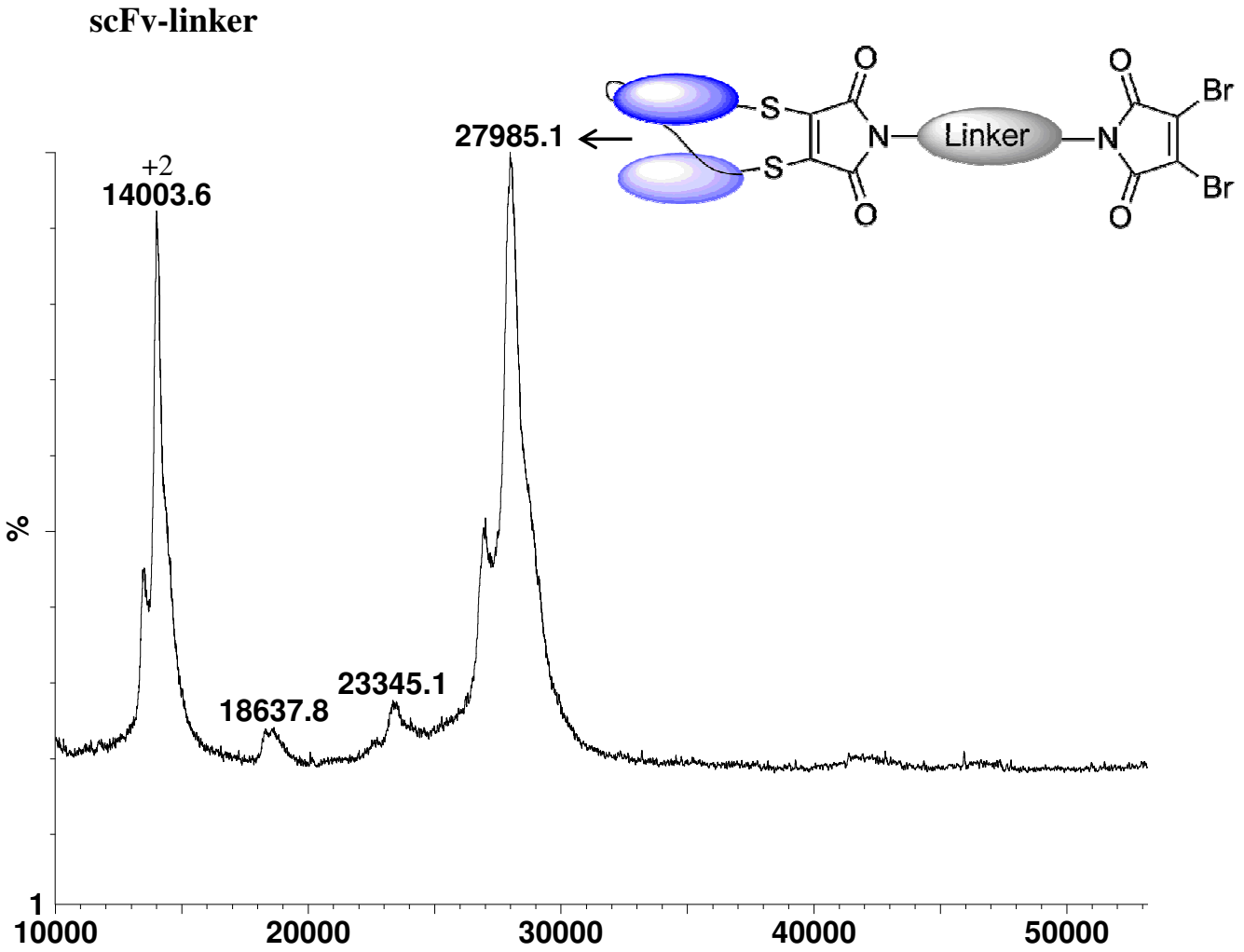



Count
0
10^0
10^1
10^2
10^3
10^4
FL 1 Log:: A488
Untreated
anti-His
anti-Human
scFv + anti-Human
Fab + anti-His

Supplementary Figure 4 – MALDI analysis of conjugates

scFv-scFv

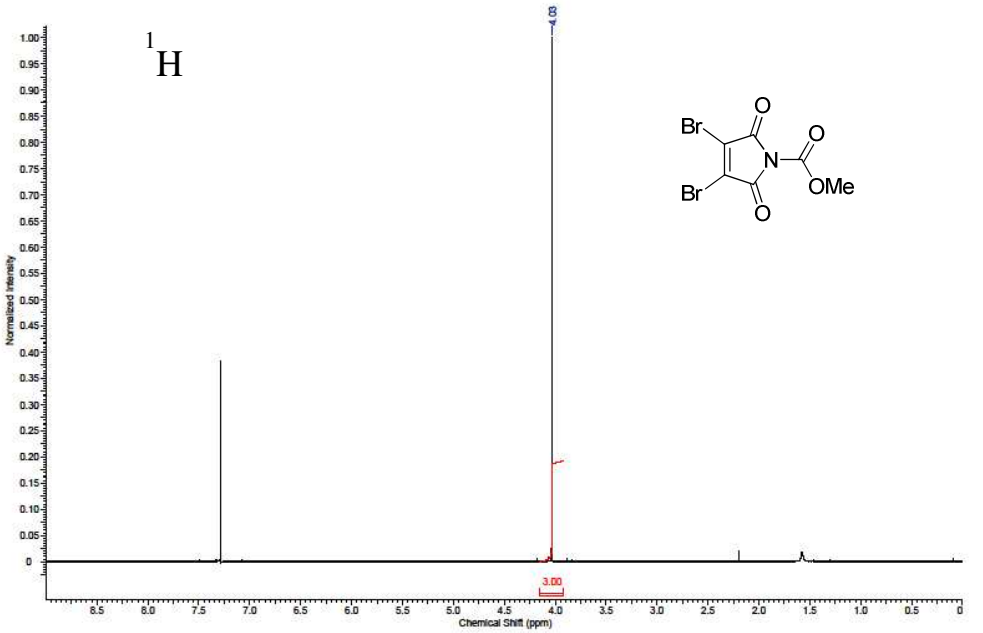


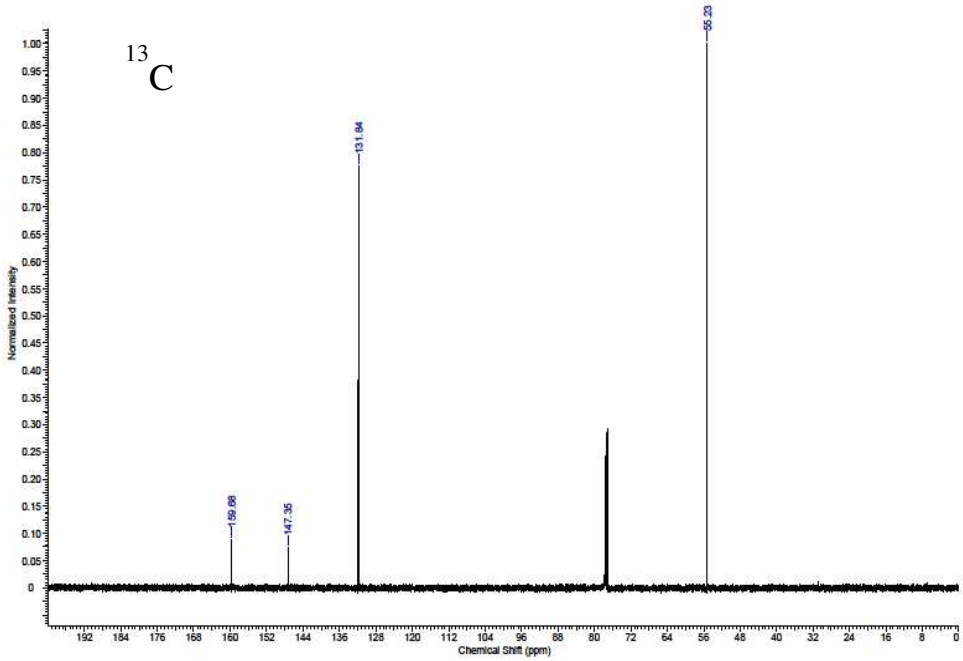


MALDI spectrum of scFv-Fab was attempted, however good peak resolution could not be achieved using the facilities available.

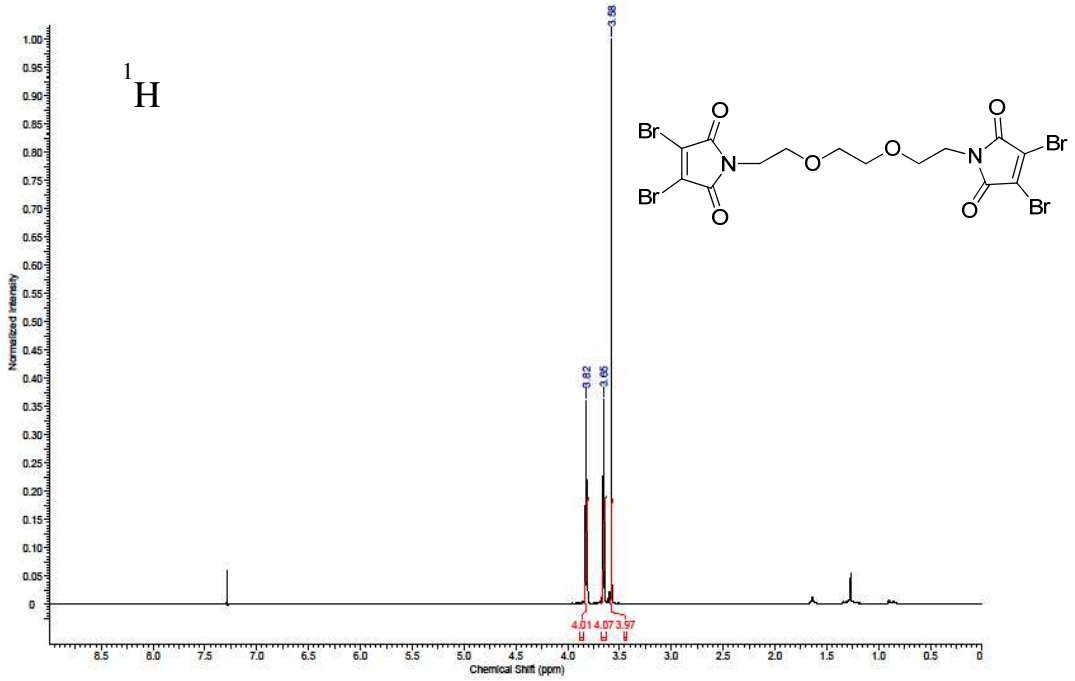
Supplementary Figure 5 – NMR spectra of synthesized compounds

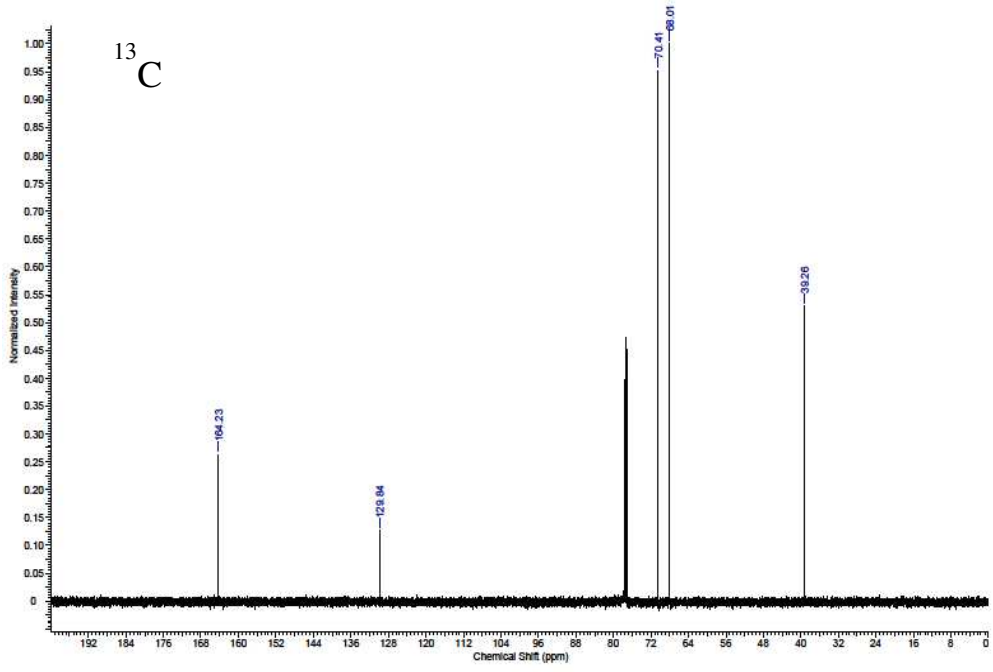
N-methoxycarbonyl-3,4-dibromomaleimide (1) [3]



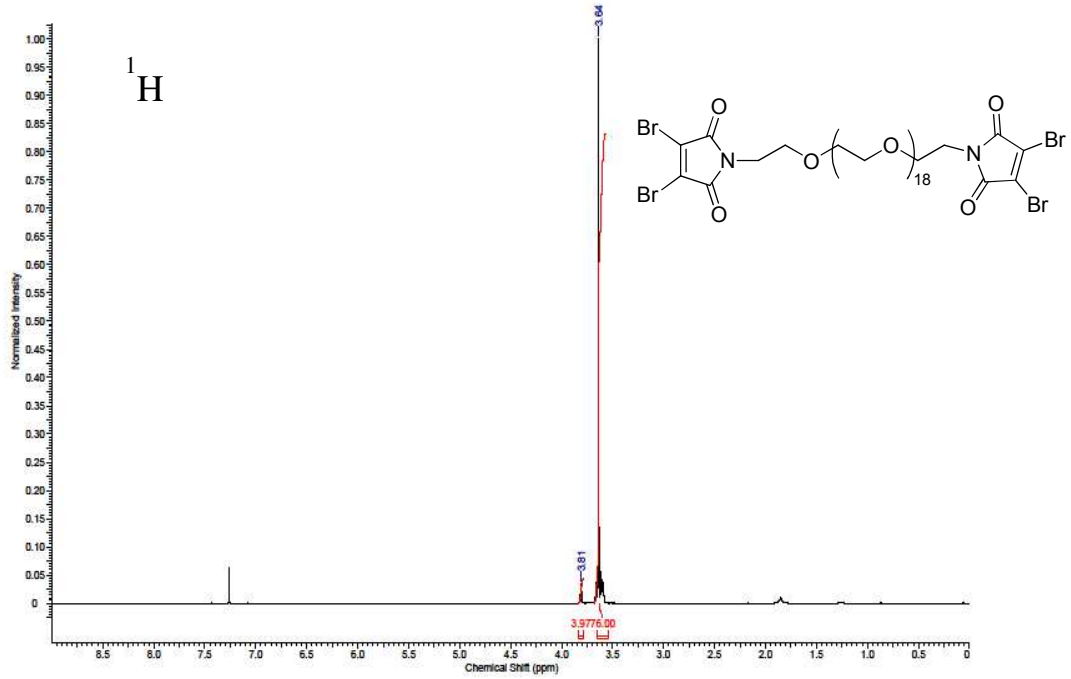


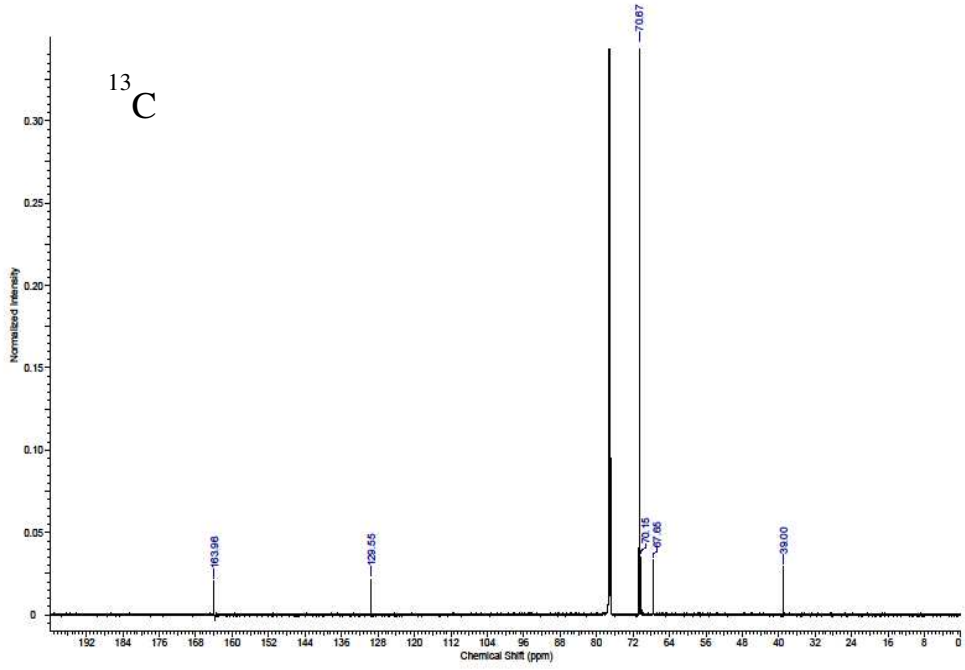
N,N-PEG2-bis-3,4-dibromomaleimide (BDBM(PEG)$_2$) [3]





N,N-PEG20-bis-3,4-dibromomaleimide (BDBM(PEG)$_{19}$)





Supplementary Methods

General Methods

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich and used without further purification. Anti-CEA ds-scFv was kindly provided by Dr Berend Tolner, UCL Cancer Institute and produced as previously described (1). Where described below petrol refers to petrol (b.p. 40-60 °C). All reactions were monitored by thin-layer chromatography (TLC) on pre-coated SIL G/UV254 silica gel plates (254 μm) purchased from VWR. Flash column chromatography was carried out with Kiesegel 60M 0.04/0.063 mm (200-400 mesh) silica gel. ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker Avance 500 or 600 instrument operating at a frequency of 500 or 600 MHz respectively for ^{1}H and 125 MHz and 150 MHz respectively for ^{13}C in a deuterated solvent as described below. 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), or multiplet (m). Coupling constants (J values) are reported in Hertz (Hz) and are H-H coupling constants unless otherwise stated. Infra-red spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer operating in ATR mode with frequencies given in reciprocal centimetres (cm^{-1}). High resolution mass data were obtained by the EPSRC UK National Mass Spectrometry Facility (NMSF), Swansea. MALDI-TOF analysis was performed on a MALDI micro MX (Micromass) by Dr Kersti Karu (UCL Mass Spectrometry Service). Melting points were measured on a Gallenkamp heating block and are not corrected. All reactions were performed in an inert-gas environment if not stated otherwise. All buffer solutions were filter-sterilised.

Trastuzumab Fab Fragment Preparation

Trastuzumab Fab was prepared by the sequential digest protocol as described previously (2).

Protocol for CEA ELISA

ELISA plates were coated with full length human CEA diluted to a final concentration of 1 μg/ml in PBS and incubated for 1 h at room temperature. After washing with PBS, the plates were blocked for 1 h at room temperature with a 5% solution of Marvel milk powder in PBS (Premier Foods). Plates were washed three times with PBS, and the serially diluted test samples (60 nM, 20 nM, 6.6 nM, 2.2 nM, 0.74 nM, 0.24 nM) of ds-scFv, Fab, scFv-scFv and scFv-Fab were added in PBS. The assay was incubated at room temperature for 1 h, washed three times with PBS-T and PBS, and the primary antibody (anti-tetra-His mouse IgG1, Quiagen, 1 : 1,000 in 1% Marvel solution) added. After 1 h the ELISA plates were washed again and the secondary antibody (ECL anti-mouse sheep IgG1 HRP linked, GE Healthcare, 1 : 1,000 in 1% Marvel solution) added and incubated for 1 h at room temperature. The plates were washed and 100 μL of 0.5 mg/mL *o*-phenylenediamine hydrochloride (Sigma-Aldrich) in a phosphate-citrate buffer with sodium perborate were added as substrate. Once colour was observed, the reaction was stopped by acidifying with 50 μL of 4 M HCl. Absorbance was immediately measured at 490 nm. Controls were included in every ELISA, in which PBS had been added to some of the wells instead of CEA or instead of antibody fragment. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average.

Protocol for HER2 ELISA

ELISA plates were coated with HER2 diluted to a final concentration of 0.25 μg/mL in PBS and incubated for 1 h at room temperature. After washing with PBS, the plates were blocked for 1 h at room temperature with a 5% solution of Marvel milk powder in PBS (Premier Foods). Plates

were washed three times with PBS, and the serially diluted test samples (60 nM, 20 nM, 6.6 nM, 2.2 nM, 0.74 nM, 0.24 nM) of ds-scFv, Fab, scFv-scFv and scFv-Fab were added in PBS. The assay was incubated at room temperature for 1 h, washed three times with PBS-T and PBS, and anti-human IgG, Fab-specific-HRP antibody (Sigma-Aldrich, 1 : 5,000 in 1% Marvel solution) was added. After 1 h the plates were washed again and 100 µL of 0.5 mg/mL *o*-phenylenediamine hydrochloride (Sigma-Aldrich) in a phosphate-citrate buffer with sodium perborate were added as substrate. Once colour was observed, the reaction was stopped by acidifying with 50 µL of 4 M HCl. Absorbance was immediately measured at 490 nm. Controls were included in every ELISA, in which PBS had been added to some of the wells instead of HER2 or instead of antibody fragment. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average.

Protocol for Sandwich ELISA

ELISA plates were coated with CEA diluted to a final concentration of 1 µg/ ml in PBS and incubated for 1 h at room temperature. After washing with PBS, the plates were blocked for 1 h at room temperature with a 5% solution of Marvel milk powder in PBS (Premier Foods). Plates were washed three times with PBS, and the serially diluted test samples (60 nM, 20 nM, 6.6 nM, 2.2 nM, 0.74 nM, 0.24 nM) of ds-scFv, Fab, scFv-scFv and scFv-Fab were added in PBS. The assay was incubated at room temperature for 1 h, washed three times with PBS-T and PBS, and HER2-Biotin (1 : 500 in 1% Marvel solution) was added. After 1 h the plates were washed again and Extravidin-Peroxidase (Sigma Aldrich, 1 : 2,000 in 1% Marvel solution) added. The plates were washed and 100 µL of 0.5 mg/mL *o*-phenylenediamine hydrochloride (Sigma-Aldrich) in a phosphate-citrate buffer with sodium perborate were added as substrate. Once colour was observed, the reaction was stopped by acidifying with 50 µL of 4 M HCl. Absorbance was

immediately measured at 490 nm. Controls were included in every ELISA. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average.

Cell Lines

The breast cancer cell lines BT474 and MDA-MB-468 were purchased from ATCC. The A375CEA was generated by stable transfection of the A375 melanoma cell line (ATCC); in brief, A375 cells were transfected with pIRES-puro-CEA (kind gift of Dr. David Gilham) and stable clones selected using 1µg/ml of puromycin (Sigma) over a period of two weeks. A375CEA and MDA-MB-468 cell lines were maintained at 37˚C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 2mM L-glutamine and complemented with 10% foetal calf serum (Labtech International, UK). BT474 cells were maintained in Hybricare X-46 (ATCC), supplemented with 10% foetal calf serum.

Flow cytometry analysis

Cell lines were prepared by removal of media and incubation with 10 ml of 0.2% EDTA for 10 min. The cells were then transferred to centrifuge tubes and pelleted (4 °C, 4 min, 1000 rpm). The EDTA solution was removed and 5 ml of fresh media added. Cells were counted and diluted to 1 million per ml, and 1 ml used for each condition to be tested. After washing with cold PBS, 200 µl of sample at 10 µg/ml was added and incubated for 1 h at 4 °C. Cells were washed with cold PBS twice, and incubated with 200 µl of detection antibody for 1 h at 4 °C. For scFv detection AlexaFluor488-conjugated Mouse anti-his tag (R&D Systems) was used and for Fab AlexaFluor488-conjugated Goat anti-human IgG (Molecular Probes). After further washing with cold PBS, the cells were suspended in 500 µl of cold PBS. Samples were analysed on a CyAn ADP High-Performance Flow Cytometer (Becton Dickinson); cells were gated according to size scattering, forward scattering and pulse width so only single cells were analysed. A total of

10,000 cell events were recorded per sample and data was analysed using FlowJo software (Tree Star Inc.).

References

(1) Schumacher, F. F., Sanchania, V. A., Tolner, B., Wright, Z. V. F., Ryan, C. P., Smith, M. E. B., Ward, J. M., Caddick, S., Kay, C. W. M., Aeppli, G., Chester, K. A., and Baker, J. R. (2013) Homogeneous antibody fragment conjugation by disulfide bridging introduces "spinostics". *Sci. Rep. 3*, 1525.

(2) Castañeda, L., Maruani, A., Schumacher, F. F., Miranda, E., Chudasama, V., Chester, K. a, Baker, J. R., Smith, M. E. B., and Caddick, S. (2013) Acid-cleavable thiomaleamic acid linker for homogeneous antibody-drug conjugation. *Chem. Commun. 49*, 8187-9.

(3) Castañeda, L., Wright, Z. V. F., Marculescu, C., Tran, T. M., Chudasama, V., Maruani, A., Hull, E. A., Nunes, J. P. M., Fitzmaurice, R. J., Smith, M. E. B., Jones, L. H., Caddick, S., and Baker, J. R. (2013) A mild synthesis of N-functionalised bromomaleimides, thiomaleimides and bromopyridazinediones. *Tetrahedron Lett. 54*, 3493-3495.