Supplementary Information (SI) for RSC Chemical Biology.
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Electronic Supporting Information

Bispecific FpFs;
A Versatile Tool for Preclinical Antibody Development

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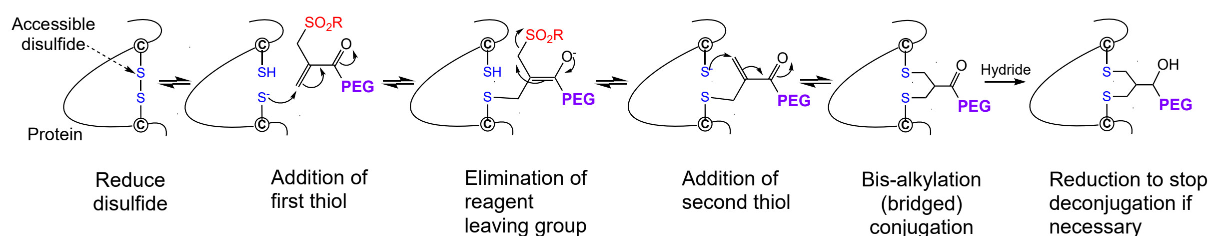
Supplementary Scheme 1

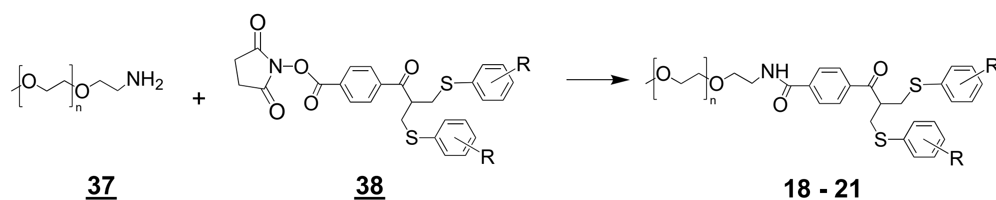


Scheme S1. Mechanism of site-specific PEGylation by bis-alkylation the thiols from a reduced, accessible protein disulfide. *Bis*-sulfone reagents (e.g. **3** and **5**) undergo elimination of the sulfinic acid leaving group as shown in **Scheme 3A** to give the *mono*-sulfone enone **9** (structure **9** in **Scheme 3A**) which can then undergo conjugation with one thiolate from the disulfide as shown above. After the first thiol addition has occurred, elimination of the second sulfinic acid leaving group can occur to produce the second enone with another α,β-unsaturated double bond to the same electron-withdrawing carbonyl for the second thiol addition reaction to occur to complete the conjugation. When necessary, mild reduction of the electron withdrawing carbonyl (e.g., $NaCNBH_3$ or $Na(AcO)_3BH$) can be conducted to avoid PEG deconjugation via a retro-Michael reaction. In practice, this is often not required as the non-covalent interactions of the protein and the flexibility of the 3-carbon bridge contribute to the stability of the bridged disulfide to prevent deconjugation. The *bis*-sulfide reagents (e.g. **18**-**21**) also undergo conjugation via an addition-elimination reaction through the formation of analogous enones (e.g. **12**, **Scheme 3A**).

Supplementary Methods

Protein concentration determination: Protein concentration was measured using UV absorbance at 280 nm. A 1.0 mg/mL solution of IgG displays an approximate absorbance of 1.39 at 280 nm with an extinction coefficient of 210,000 $M^{-1}cm^{-1}$. A Fab fragment at a concentration of 1.0 mg/mL has an absorbance of approximately 1.40 at 280 nm with an extinction coefficient of 216,000 M^{-1} cm^{-1}. This latter value was confirmed with a solution of ranibizumab, a Fab molecule, (1.0 mg/mL) that was prepared from the pharmaceutical formulation (10 mg/mL). As the linker in the PEG reagent contributes to the UV absorbance at 280 nm, the bicinchoninic acid (BCA) assay was used for PEGylated protein samples while UV spectroscopy at 280 nm was used for most unPEGylated protein samples.

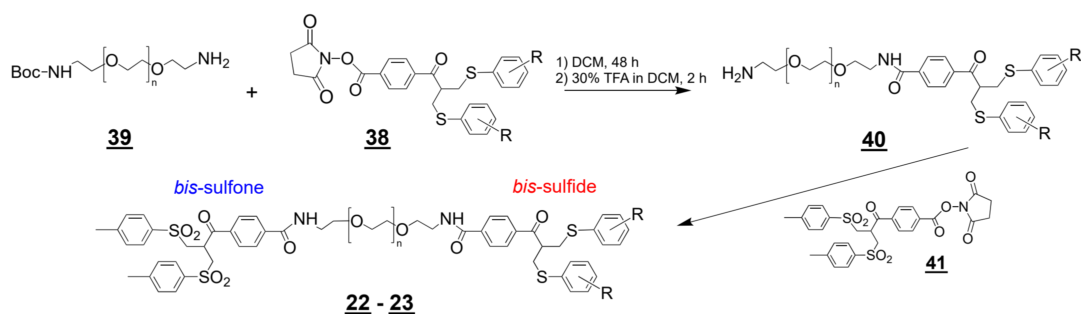
*Preparation of PEG_{10} bis-sulfides **18** - **21***



As a representative procedure [1, 2], methoxy-PEG_{10}-NH_2 **37** (10 kDa; 1 g, 1 × 10^{-4} mole, 1 equiv.) was placed in a single neck round bottom flask (250 mL) and dissolved in toluene (5.0 mL), which was then evaporated to remove residual water as an azeotrope. The solid PEG amine **37** was re-dissolved in anhydrous DCM (2.5 mL) and *bis*-sulfide-*o*-fluoride N-hydroxysuccinimide (NHS) ester **38** (0.32 g, 6 × 10^{-4} mole, 6 equiv.; prepared as described in [1]**)** was dissolved in DCM (2.5 mL) and added drop-wise to the reaction mixture which was then allowed to incubate for 24-48 h at ambient temperature. The DCM was removed by rota-evaporation and the residue dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and the desired product (75-85%) was precipitated (3×) from acetone that had been chilled over dry ice (as described in [3]).

^{1}H NMR for PEG-*bis*-sulfide-*o*-fluoride **19** ($CDCl_3$, 400 MHz): δ (ppm) 3.10-3.30 (m, 4H, C**H**$_2$S*Ar*), 3.35 (s, 3H, PEG-OC**H**$_3$), 3.40-3.80 (br, PEG), 4.05-4.10 (m, 1 H, C**H**C=O), 6.90-7.00 (m, 4H, SF*Ar*CH_2C**H** and SF*Ar* CH_2CH_2C**H**), 7.15-7.20 (dd, SF*Ar*C**H** and SF*Ar* $CH_2CH_2CH_2$C**H**), 7.55-7.60 (d, 2H, C=O*Ar*C**H**), 7.70-7.75 (d, 2H, *Ar*C**H**C=O).

Preparation bis-sulfide bis-sulfone reagents ***22*** *-* ***23***



As a representative procedure, the synthesis of the *ortho*-fluoro *bis*-sulfide *bis*-sulfone reagent **22** [1]. Boc-NH-PEG_{10}-NH_2 **39** (Iris Biotech Gmbh, 10 kDa, 1 g, 1 × 10^{-4} mole, 1 equiv.) was dissolved in toluene (5.0 mL) in a round bottom flask and the toluene was evaporated in vacuum to remove residual water. The solid Boc-NH-PEG_{10}-NH_2 **39** was re-dissolved in anhydrous DCM (2.5 mL). In a separate vial *bis*-sulfide-*o*-fluoride N-hydroxysuccinimide (NHS) ester **38** (0.32 g, 6 × 10^{-4} mole, 6 equiv.; prepared as described in [1]) was dissolved in anhydrous DCM (2.5 mL) and added drop-wise to the Boc-NH-PEG_{10}-NH_2 **39** solution. The reaction mixture was sealed with a septum and allowed to stir for 48 hours at RT (~25°C). After roto-evaporation of the DCM, the residue dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and precipitated (3×) from acetone chilled over dry ice (as described in [3]). The residue was dried by vacuum and then dissolved in 30% TFA in anhydrous DCM (2.0 mL). After 2-3 h, the DCM and most of the TFA were removed by roto-evaporation and then vacuum. The residue was then dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and precipitated (3×) from acetone chilled over dry ice (as described in [3]) to give H_2N-PEG_{10}-bis sulfide **40** (50-60% yield from Boc-NH-PEG_{10}-NH_2 **39**).

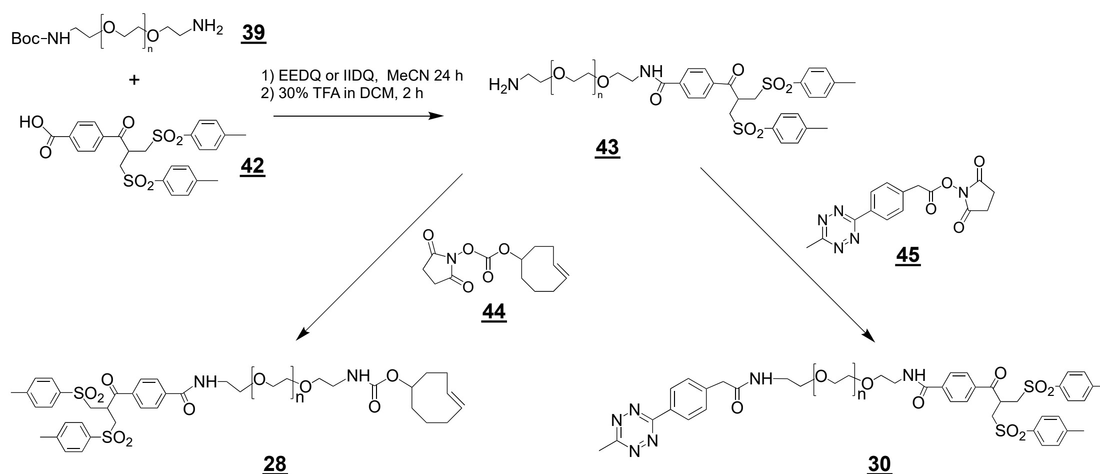
^{1}H NMR ($CDCl_3$, 400 MHz) intermediate **40**: δ (ppm) 2.28 (s, 6H, ArC**H**$_3$), 3.01-3.20 (m, 4H, C**H**$_2$SAr), 3.40-3.80 (br, PEG), 6.92 (br, 1H, PEGN**H**C=O), 6.97 (d, 4H, *Ar*C**H**CH_3), 7.12 (d, 4H, S*Ar*C**H**), 7.51 (d, 2H, C=O*Ar*C**H**), 7.70 (d, 2H, *Ar*C**H**C=O).

To *bis*-sulfide PEG amine **40** (10 kDa, 0.6 g, 6 × 10^{-5} mole, 1 equiv.) dissolved in DCM (2.5 mL) in a single neck round bottom flask was added dropwise a DCM solution (2.5 mL) of *bis*-sulfone N-hydroxysuccinimide (NHS) ester **41** (0.21 g, 3.6 × 10^{-4} mole, 6 equiv.; prepared as described in [1]). The reaction mixture was allowed to stir for 48 hours at RT (~25°C). The DCM was removed by rota-evaporation and the residue dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and

the desired product (~80-90%) was precipitated (3×) from acetone that had been chilled over dry ice (as described in [3]).

^{1}H NMR ($CDCl_3$, 400 MHz) *ortho*-fluoro *bis*-sulfide *bis*-sulfone reagent **22**: δ (ppm) 2.28 (s, 6H, SArC**H**$_3$), 2.41 (s, 6H, SO_2ArC**H**$_3$), 3.02 (m, 4H, C**H**$_2$SAr), 3.10 (m, 4H, C**H**$_2$$SO_2$Ar), 3.40-3.80 (br, PEG), 4.29 (br, 1H,SO_2CH_2C**H**), 6.92 (br, 1H, PEGN**H**), 6.99 (d, 4H, S*Ar*C**H**CH_3), 7.05 (d, 4H, S*Ar*C**H**CHCH_3), 7.30 (d, 4H, SO_2*Ar*C**H**CH_3), 7.55 (d, 4H, SO_2*Ar*C**H**CHCH_3), 7.65 (d, 4H, ArC**H**C=ONH), 7.76 (dd, 4H, *Ar*C**H**CHC=ONH).

*Preparation of cyclooctene (TCO) and tetrazine (Tz) bis-sulfone reagents **28** and **30***



The *bis*-sulfone carboxylic acid **42** (prepared as described in [2]) was coupled directly to Boc-NH-PEG-NH_2 **39** (5 and 10 kDa) using either N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) or isobutyl 1,2-dihydro-2-isobutoxy-1-quinolinecarboxylate (IIDQ) [4]. As a representative procedure, to an acetonitrile solution (2.0 mL) of Boc-NH-PEG_{10}-NH_2 **39** (10 kDa, 0.4 g, 4.0×10^{-5} mole, 1 equiv.) and *bis*-sulfone carboxylic acid **42** (0.1 g, 2.0×10^{-4} mole, 5 equiv.) was added dropwise IIDQ (72 mg, 2.4×10^{-4} mole, 6 equiv., Sigma Aldrich) in an acetonitrile (1.0 mL). After the reaction mixture was gently stirred for 24 h, the acetonitrile was removed by evaporation under reduced pressure and the residue dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and precipitated (3×) from acetone chilled over dry ice (as described in [3]). The residue was dried by vacuum and then dissolved in 30% TFA in anhydrous DCM (2.0 mL) and stirred for 2-3 h after which the DCM and most of the TFA were removed by roto-evaporation and then vacuum. The residue was then dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and precipitated (3×) from acetone chilled over dry ice (as described in [3]) to give H_2N-PEG_{10}-*bis*-sulfone **43** (80-90% yields typically from Boc-NH-PEG-NH_2 **39**, 5 and 10 kDa PEG precursors).

^{1}H NMR ($CDCl_3$, 400 MHz) intermediate **43**: δ (ppm) 2.49 (s, 6H, ArC**H**$_3$), 3.40-3.80 (m, 4H, C**H**$_2$SAr; br, PEG), 4.35 (m, 1H, C**H**C=O), 6.97 (d, 4H, *Ar*C**H**CH_3), 7.36, 7.69 (2 superimposed AB q, 4H, SO_2Ar**H**Me, 4H, COAr), 7.64, 7.81 (AB q, 4H, SO_2**H**ArMe).

As a representative procedure, the TCO *bis*-sulfone reagent **28** was prepared by allowing a DCM solution (18.0 mL) of *bis*-sulfone PEG_{10} amine **43** (10.5 kDa g/mol, 0.39 g, 3.8×10^{-5} mole, 1 equiv.), TCO NHS ester **44** (Iris Biotech Gmbh, 0.04 g, 1.5×10^{-5} mole, 4 equiv.) and N-methyl morpholine (NMM) (0.92 g/mL,15.0 mg, 1.4×10^{-4} mole, 4 equiv.) to stir for 24 h at ambient temperature. Although dimethylamino pyridine (DMAP) can be used in place of NMM, DMAP is a stronger base than NMM and can cause some elimination of sulfinic acid, which is not deleterious, but requires more careful handling of the conjugation reagents. The DCM was evaporated at reduced pressure and the residue dissolved in minimal acetone (~20 mg solid per mL of acetone) and the desired product **28** (typically 60-70%) was precipitated (3-5×) from acetone that had been chilled over dry ice (as described in [3]).

In analogous fashion, the Tz *bis*-sulfone reagent **30** was prepared by allowing a DCM solution (18.0 mL) of *bis*-sulfone PEG_{10} amine **43** (10.5 kDa g/mol, 0.18 g, 1.7×10^{-5} mole, 1 equiv.), Tz NHS ester **45** (Iris Biotech Gmbh, 0.018 g, 5.2×10^{-5} mole, 3 equiv.) and N-methyl morpholine (NMM) (0.92 g/mL,15.0 mg, 1.4×10^{-4} mole, 8 equiv.) to stir for 24 h at ambient temperature. The DCM was evaporated at reduced pressure and the residue dissolved in minimal acetone (~15-20 mg solid per mL of acetone) and the desired product **30** (typically 60-70%) was precipitated (4-6 ×) from acetone that had been chilled over dry ice (as described in [3]). Effort was made to ensure the supernatant from the precipitation was colourless.

^{1}H NMR ($CDCl_3$, 400 MHz) *bis*-sulfone-PEG-TCO **28**: δ (ppm) 1.65-2.00 (m, 6H, C**H**$_2$), 2.28 (s, 6H, ArC**H**$_3$),2.28-2.39 (m, 4H, C**H**$_2$), 3.40-3.80 (m, 4H, C**H**$_2$SAr; br, PEG), 4.35 (m, 1H, C**H**C=O, m 1 H OC**H**(CH_2)$_2$), 6.97 (d, 4H, *Ar*C**H**CH_3), 7.36, 7.69 (2 superimposed AB q, 4H, SO_2Ar**H**Me, 4H, COAr), 7.64, 7.81 (AB q, 4H, SO_2**H**ArMe).

^{1}H NMR ($CDCl_3$, 400 MHz) *bis*-sulfone-PEG-Tz **30**: δ (ppm) 2.49 (s, 6H, ArC**H**$_3$), 3.04 (s, 3H, tetrasolAr-C**H**$_3$) 3.40-3.80 (m, 4H, C**H**$_2$SAr; br, PEG), 4.35 (m, 1H, C**H**C=O), 6.97 (d, 4H, *Ar*C**H**CH_3), 7.36, 7.69 (2 superimposed AB q, 4H, SO_2Ar**H**Me, 4H, COAr), 7.64, 7.81 (AB q, 4H, SO_2**H**ArMe) 7.50, 8.50 (AB q, 4H, tetrazol-Ar**H**).

Supplementary Figures

Figure S1

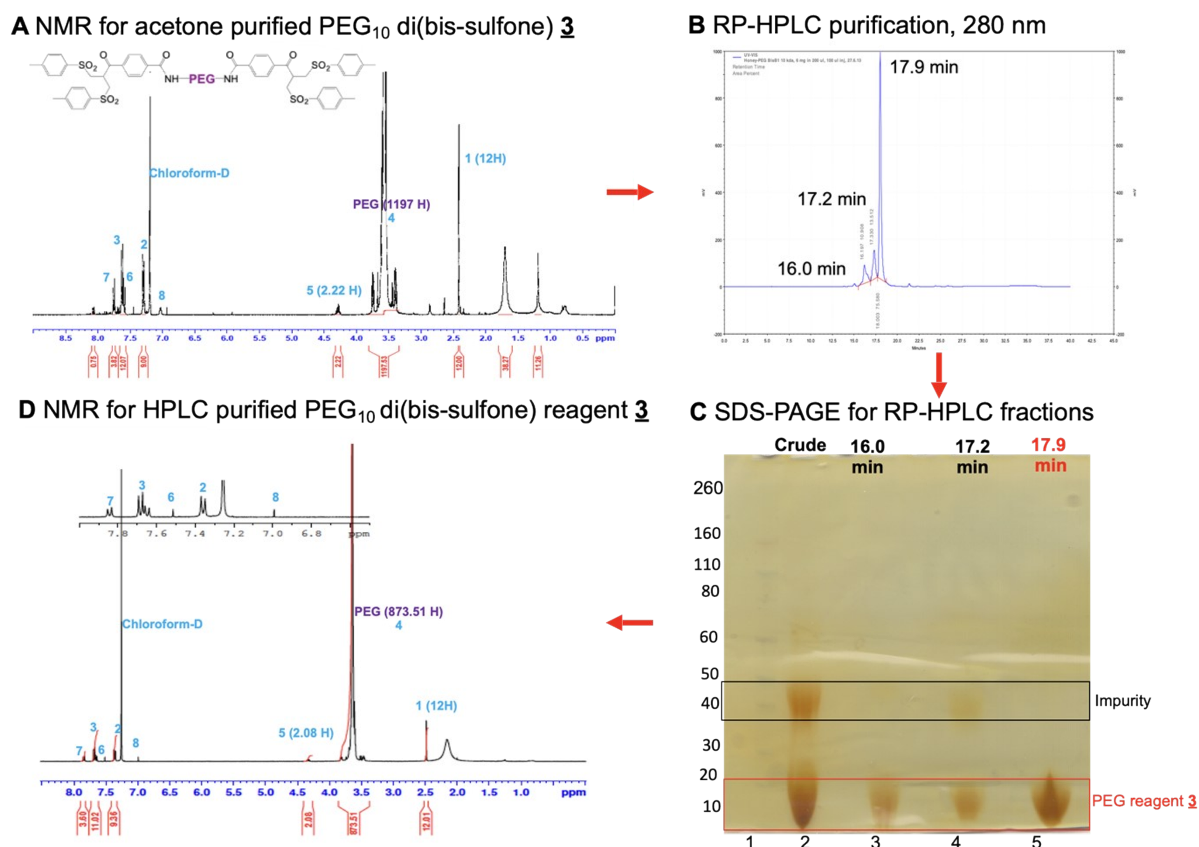


Figure S1. (A) ^{1}H-NMR analysis of the PEG_{10} di(bis-sulfone) **3** after acetone precipitation purification. ^{1}H NMR: (CDCl3, 400 MHz) δ 2.49 (s, 12H, CH3Ar), 3.38 (s, 6H, CH3OPEG), 3.44-3.84 (m, PEG + 4H, CH2SO2), 4.34 CHCO (qn, 2H, CHCO), 7.36, 7.69 (AB q, SO2Ar, 8H, J = 16.6 MHz), 7.64, 7.81 (AB q, COAr, 8H, J = 8.3 MHz). PEG_{10} protons appeared between ~3.4-4.5 ppm with an integration of 1197.53 bigger than theoretical integration value of ~900 suggesting an excess unreacted PEG di(amine) within the mixture. **(B)** Reverse phase-HPLC analysis of PEG_{10} di(bis-sulfone) **3** at A280 nm, 150 mg scale. Three peaks appeared at 16.0, 17.2 and 17.9 min. N=20 samples), **(C)** SDS-PAGE analysis of HPLC fractions stained with barium iodide staining, ***lane 1:*** Novex pre-stained marker, ***lane 2:*** PEG reagent **3** after acetone precipitation purification and before RP-HPLC, ***lane 3:*** RP-HPLC fraction at 16 min, ***lane 4:*** RP-HPLC fraction at 17.2 min and ***lane 5,*** RP-HPLC fraction at 17.9 min, **(D)** ^{1}H-NMR analysis of the PEG_{10} di(bis-sulfone) **3** after HPLC purification (fraction collected at 17.9 min), PEG_{10} protons appeared with an integration of 873.51, close to the theoretical integration value of ~900.

Figure S2

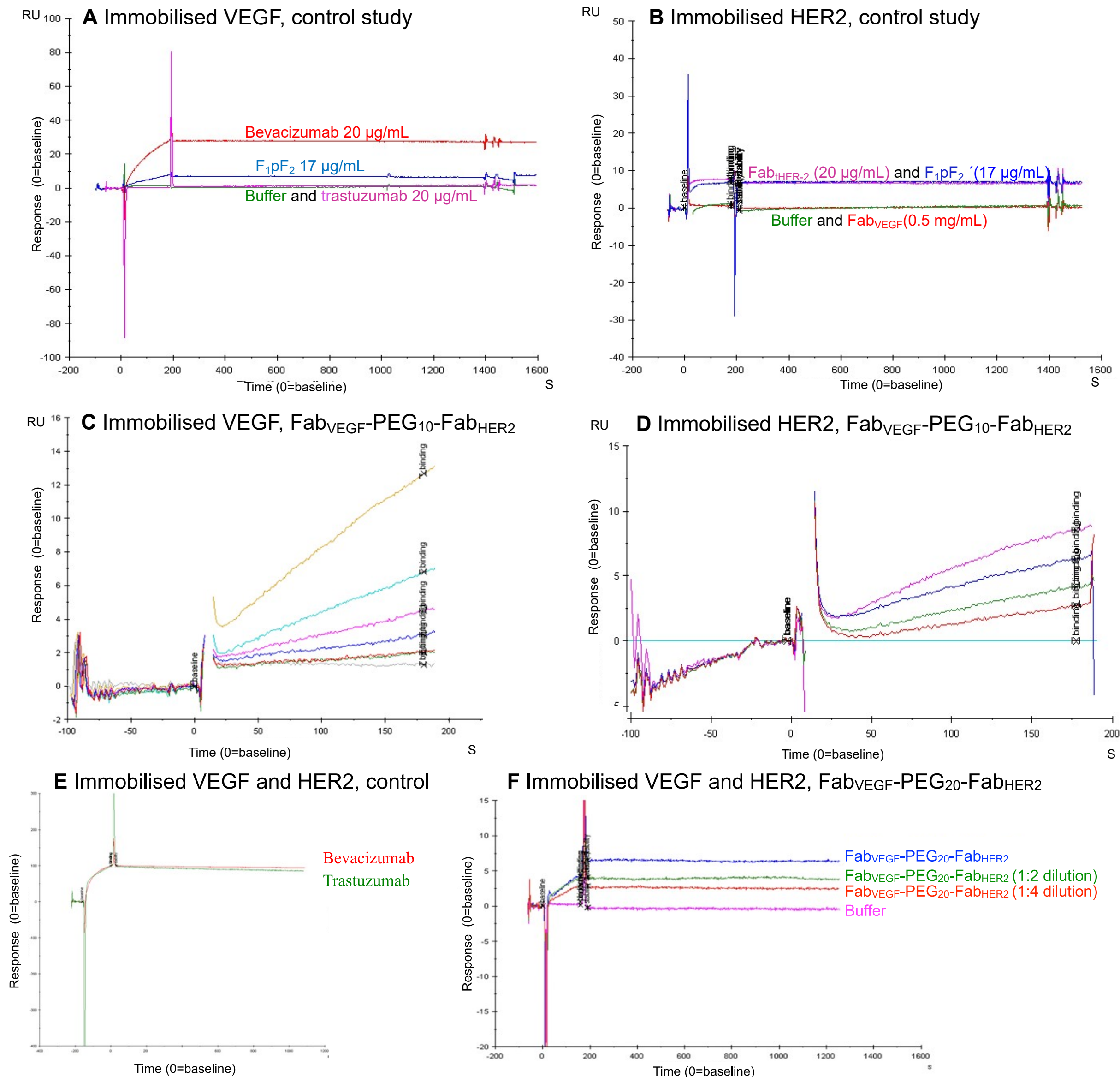


Figure S2. Superposition of sensograms obtained for Fab_{VEGF}-PEG_{10}-Fab_{HER2}. A control study was performed using bevacizumab, trastuzumab and Fab_{VEGF}-PEG_{10}-Fab_{HER2} with **(A)** a CM3 chip immobilised with VEGF (55 RU) and **(B)** a CM3 chip immobilised with HER2 (65 RU). Trastuzumab and Fab_{VEGF} (obtained from bevacizumab) displayed binding to the VEGF chip and HER-2 chip respectively. This suggested that binding of Fab_{VEGF}-PEG_{10}-Fab_{HER2} to VEGF and HER-2 was specific. Binding sensograms for Fab_{VEGF}-PEG_{10}-Fab_{HER2} were obtained at various concentrations (1.06-17.00 μg/mL) using **(C)** a CM3 chip immobilised with VEGF (55 RU) and **(D)** another CM3 chip immobilised with HER-2 (65 RU). The bispecific Fab_{VEGF}-PEG_{10}-Fab_{HER2} **2** conjugate displayed binding to both immobilised ligands in concentration-dependent manner. **(E)** Bevacizumab (targeting VEGF) and trastuzumab (targeting HER2) both display binding sensograms for a chip with both immobilised VEGF and HER2 **(F)** Fab_{VEGF}-PEG_{20}-Fab_{HER2} displays binding in a concentration-dependent manner to a chip with both immobilised VEGF and HER2.

Figure S3

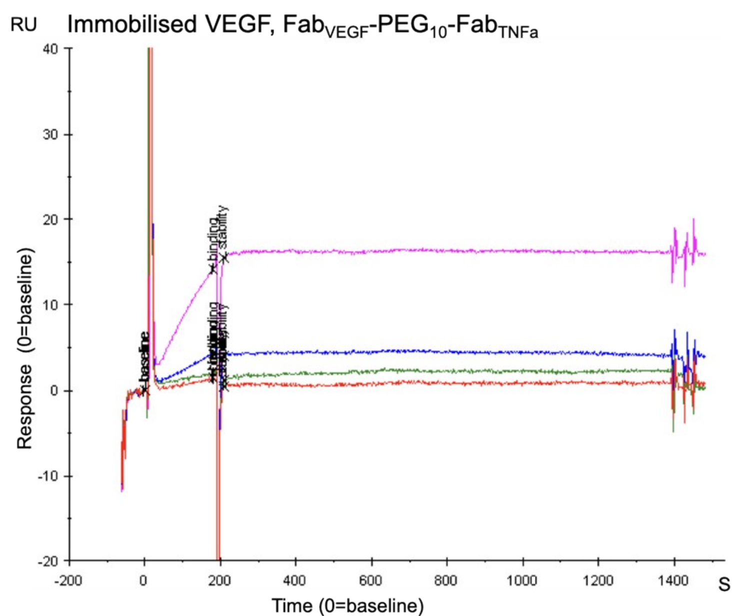


Figure S3. Binding sensograms of Fab_{VEGF}-PEG_{10}-$Fab_{TNF\text{-}\alpha}$ **2** obtained at different concentrations (2.5 to 20 µg/mL) using a CM3 chip immobilised with VEGF (55 RU). The bispecific Fab_{VEGF}-PEG_{10}- $Fab_{TNF\text{-}\alpha}$ **2** demonstrated concentration-dependent binding.

Figure S4

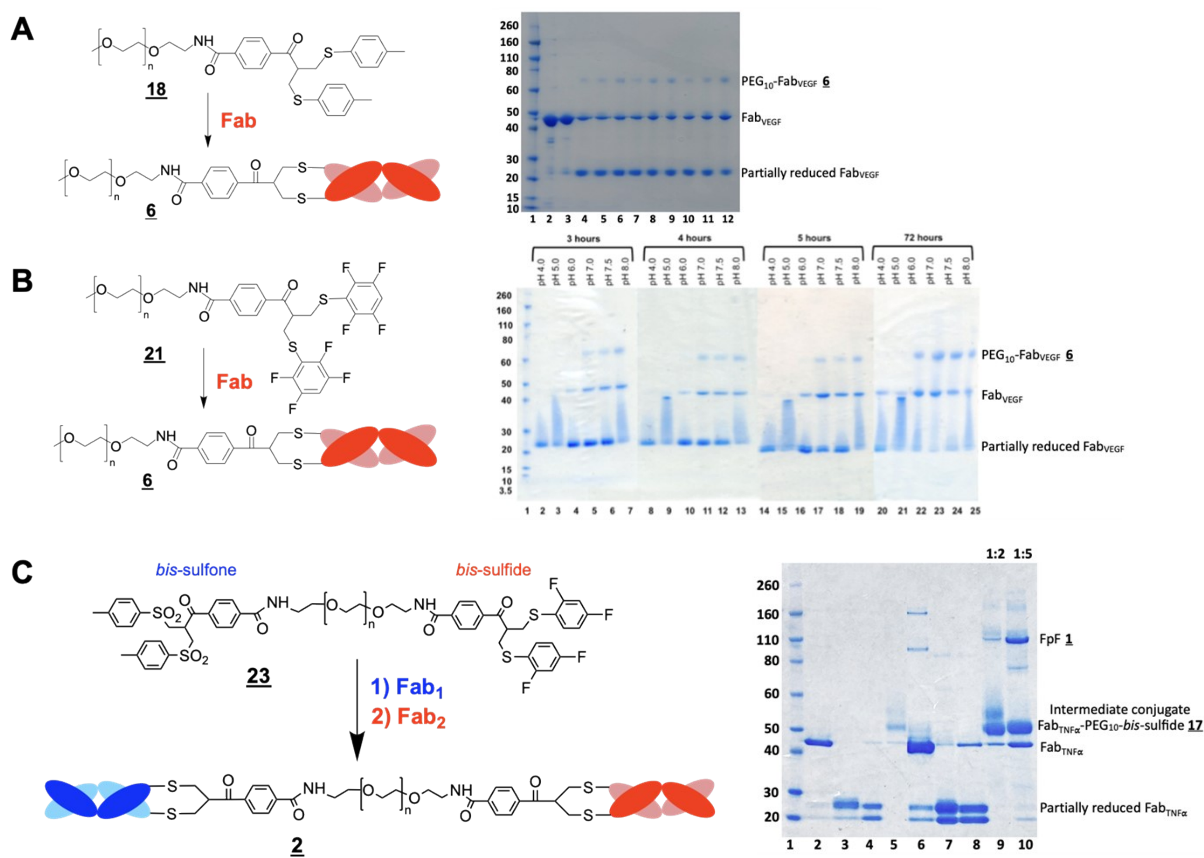


Figure S4. (A) SDS PAGE gel of the conjugation of 0.3 mg ranibizumab per conjugation (0.30 mg/mL) with increasing stoichiometries to PEG$_{10}$ *bis*-sulfide 18 from 1.5 to 2.5 equivalents at pH 8.2 for 2, 3 and 18 hours conjugation times – Coomassie blue: ***lane 1*:** Protein markers, ***lane 2***: ranibizumab, ***lane 3**:* reduced ranibizumab, ***lanes 4-6**:* 1.5 equiv. 18 for 2, 3 and 18 h respectively; ***lanes 7-9**:* 2 equiv. 18 for 2, 3 and 18 h respectively; ***lanes 10-12**:* 2.5 equiv. 18 for 2, 3 and 18 h respectively. **(B)** Scouting conjugation of Fab$_{RANI}$ to PEG$_{10}$-bis-sulfide *tetra*-fluoride 21 (2 eq) from pH 4-8 at different incubation times. ***Lane 1:*** protein markers, ***lanes 2–7:*** 3-hour incubation time, ***lanes 8–13:*** 4-hour incubation time, ***lanes 14–19:*** 5-hour incubation time and ***lanes 20-25:*** 72-hour incubation time. **(C)** Scouting reaction to prepare a FpF in two steps using di-fluoro *bis*-sulfide *bis*-sulfone reagent 23. The same Fab$_{TNF\alpha}$ was used in both conjugation steps so a FpF 1 was prepared rather than a bsFpF 2. Fab$_{TNF\alpha}$ was obtained from the proteolytic digestion of infliximab. Novex Bis-Tris 4-12% gel stained with Coomassie Blue: ***lane 1:*** protein standard, ***lane 2:*** Fab$_{TNF\alpha}$, ***Lane 3:*** reduced Fab$_{TNF\alpha}$ (pH 6.0), ***lane 4:*** reduced Fab$_{TNF\alpha}$ after DTT removal by elution over a PD10 column (pH 6.0), ***lane 5:*** conjugation of Fab$_{TNF\alpha}$ to give the Intermediate conjugate (Fab$_{TNF\alpha}$-PEG$_3$-*bis* sulfide), ***lane 6:*** Fab$_{TNF\alpha}$ sample used for the second conjugation step, ***Lane 7:*** reduced Fab$_{TNF\alpha}$ for the second conjugation step (pH 8.0), ***Lane 8:*** Reduced Fab$_{TNF\alpha}$ after DTT removal, ***Lane 9:*** conjugation of Fab$_{TNF\alpha}$ (2 equivalents) to the intermediate conjugate, Fab$_{TNF\alpha}$-PEG$_3$-*bis* sulfide to give Fab$_{TNF\alpha}$-PEG$_3$-Fab$_{TNF\alpha}$ 1, ***Lane 10:*** conjugation of Fab$_{TNF\alpha}$ (5 equivalents) to the intermediate conjugate, Fab$_{TNF\alpha}$-PEG$_3$-*bis* sulfide to give Fab$_{TNF\alpha}$-PEG$_3$-Fab$_{TNF\alpha}$ 1. Increasing the stoichiometric ratio of the Fab for the second conjugation improved the conversion to the desired FpF.

Figure S5

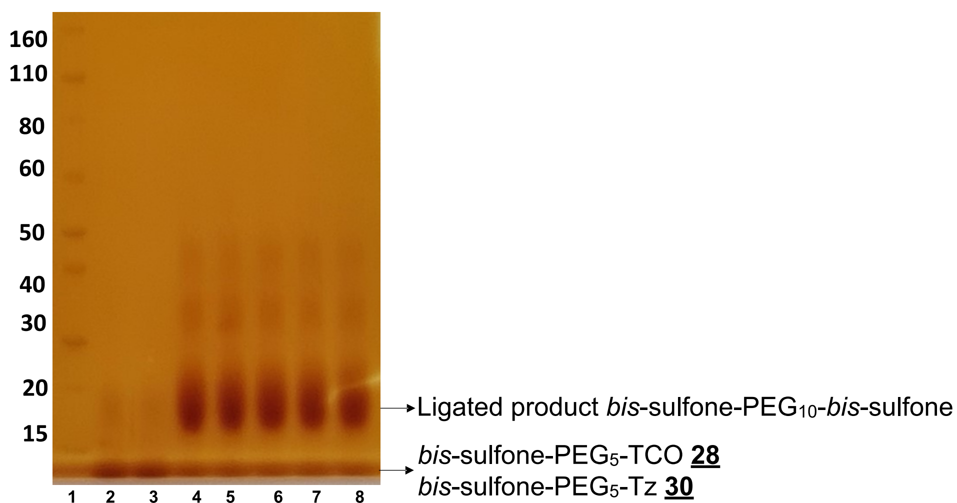


Figure S5. SDS PAGE gel showing ligation of *bis*-sulfone-PEG$_5$-TCO **28** and *bis*-sulfone-PEG$_5$-Tz **30**,1:1 molar ratio stained with barium iodide at a range of different pHs, 2-hour reaction time. ***lane 1:*** Protein marker, ***lane 2:*** *bis*-sulfone-PEG$_5$-TCO **28**, ***lane 3:*** *bis*-sulfone-PEG$_5$-Tz **30**, ***lanes 4-8:*** reagents **28** and **30** ligated at pH 5, ***lane 5:*** at pH 6, ***lane 6:*** at pH 7, ***lane 7:*** at pH 8, ***lane 8:*** at pH 9.

Figure S6

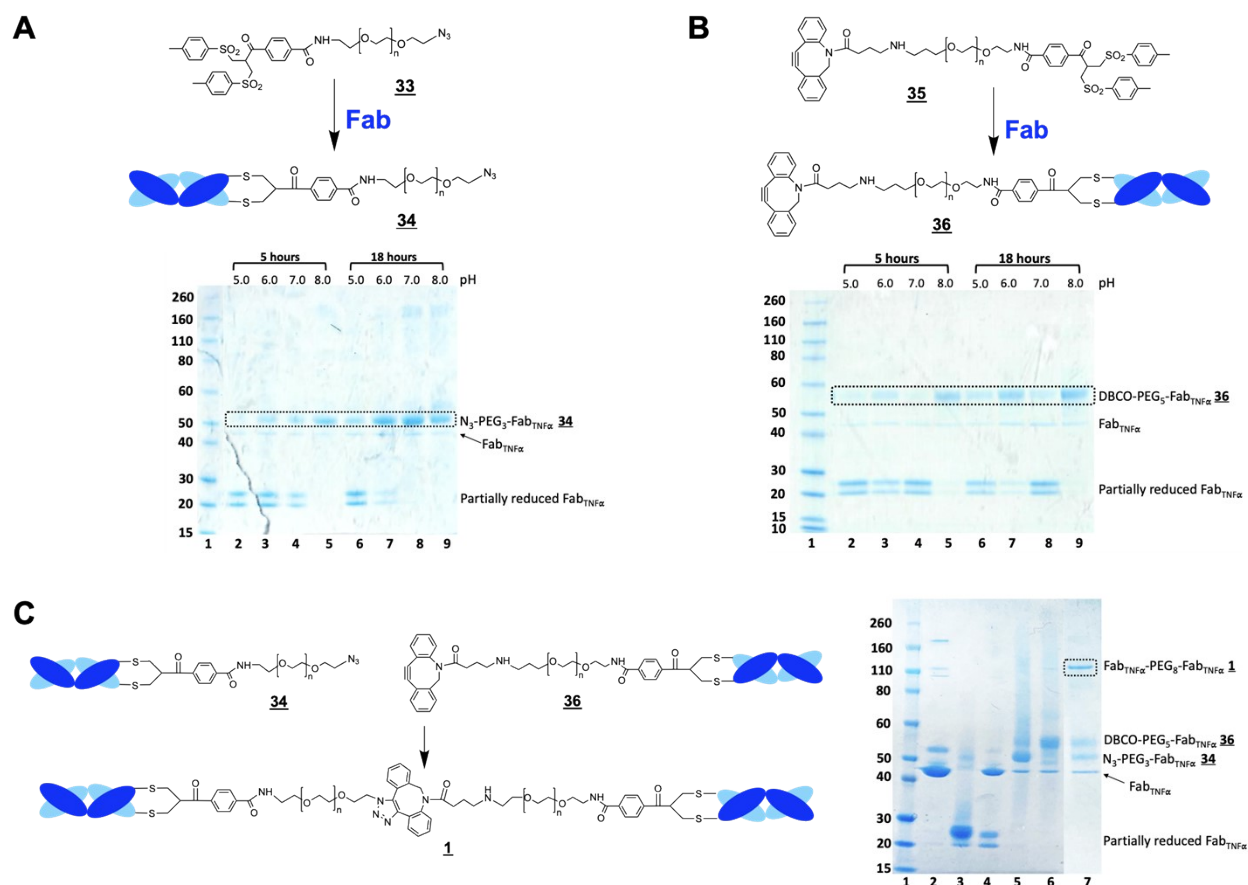


Figure S6. Conjugation scouting reactions using 2 equivalents of either **(A)** *bis* sulfone-PEG$_3$-N$_3$ **33** and **(B)** *bis* sulfone-PEG$_5$-DBCO **35**. Novex Bis-Tris 4-12% gel stained with Coomassie Blue; ***lane 1:*** protein marker, ***lanes 2 – 5***: Fab$_{TNF\alpha}$ pH scouting reactions 5-hour incubation time, ***lanes 6 – 9***: Fab$_{TNF\alpha}$ pH scouting reactions 18-hour incubation time. **(C)** Ligation of Fab$_{TNF\alpha}$ conjugates **34** and **36**; ***lane 1:*** protein standard, ***lane 2:*** Fab$_{TNF\alpha}$, ***lane 3:*** reduced Fab$_{TNF\alpha}$ (pH 8.0), ***lane 4:*** reduced Fab$_{TNF\alpha}$ after DTT removal by PT10 elution (pH 8.0), ***lane 5:*** conjugation mixture (pH 8.0, 5-hour incubation) for the preparation of Fab$_{TNF\alpha}$-PEG$_3$-N$_3$ **34**, ***lane 6:*** conjugation mixture (pH 8.0, 5-hour incubation) for the preparation of Fab$_{TNF\alpha}$-PEG$_5$-DBCO **36**, ***lane 7:*** Fab$_{TNF\alpha}$-PEG$_8$-Fab$_{TNF\alpha}$ using a 1:1 ratio of conjugates **34** and **36**.

Figure S7

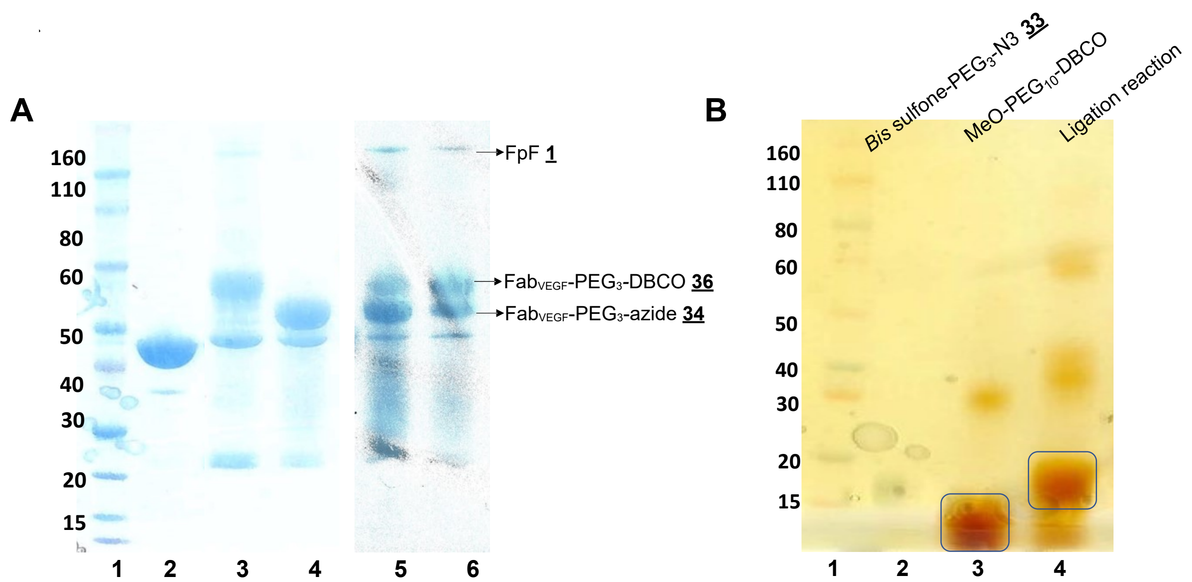


Figure S7. (A) SDS PAGE gel showing the conjugation of Fab_{VEGF} with *bis*-sulfone-PEG_3-N_3 **33** and *bis*-sulfone-PEG_{10}-DBCO **35** followed by ligation. ***Lane 1:*** Protein marker, ***lane 2***: Fab_{VEGF} , ***lane 3***: Fab_{VEGF}-PEG_3-N_3 conjugate intermediate **34**, ***lane 4***: Fab_{VEGF}-PEG_{10}-DBCO conjugate intermediate **36**, ***lanes 4, 5***: Ligation reaction between conjugate intermediates **34** and **36** to obtain the FpF **1**. **(B)** SDS PAGE gel showing the ligation of commercially sourced Me-PEG_{10}-DBCO with PEG_3 *bis*-sulfone azide at a 1:1 molar ratio stained with barium iodide. ***lane 1:*** Protein marker, ***lane 2***: *bis*-sulfone-PEG_3-azide **33**, ***lane 3***: commercially purchased Me-PEG_{10}-DBCO, ***lane 4***: ligation reaction between *bis*-sulfone-PEG_3-azide **33** and Me-PEG_{10}-DBCO.

Figure S8

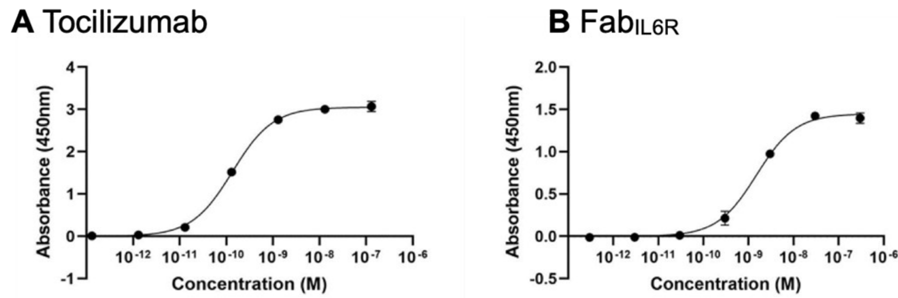


Figure S8. ELISA curves for **(A)** Tocilizumab and **(B)** Fab_{IL6R} with a concentration range of 1.3 x 10^{-7} to 1.3x 10^{-13} M over wells coated with IL6R (0.1 µg).

Figure S9

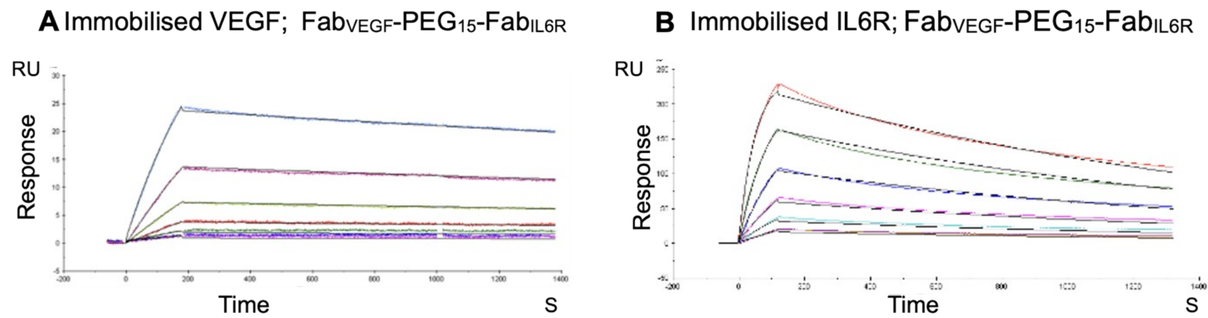


Figure S9. SPR binding sensograms for **(A)** bispecific Fab_{VEGF}-PEG_{15}-Fab_{IL6R} using CM3 chip immobilised with VEGF (95 RU), and **(B)** bispecific Fab_{VEGF}-PEG_{15}-Fab_{IL6R} using NTA chip functionalised with IL6R (his-tag).

Figure S10

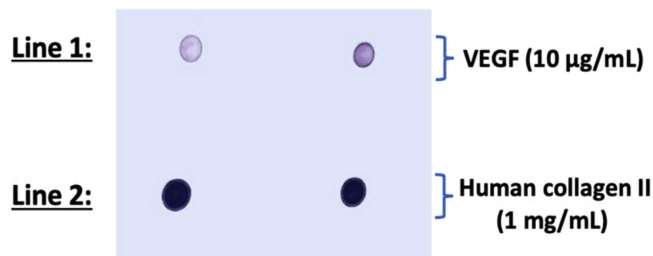


Figure S10. Immunoblotting (dot blot) assay for *Fab_{VEGF}-PEG_{15}-Fab_{COL2}* to VEGF (Line 1) and human collagen II (Line 2). The appearance of the dark spots is an indication of binding of the heterodimer to both ligands.

Figure S11

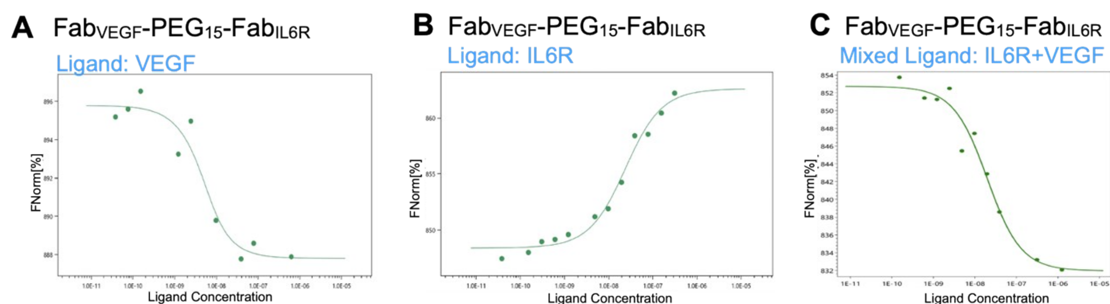


Figure S11: **(A)** MST curve of the Fab_{VEGF}-PEG_{15}-Fab_{IL6R} (5nM) binding to VEGF (1.09×10^{-7} - 3.31×10^{-11}M), **(B)** MST curve of the Fab_{VEGF}-PEG_{15}-Fab_{IL6R} (5nM) binding to IL6R (6.25×10^{-7} to 3.81×10^{-11}M) **(C)** MST curve of the Fab_{VEGF}-PEG_{15}-Fab_{IL6R} (5nM) binding to mixed VEGF and IL-6R (1:1 molar ratio). Combined ligand concentrations ranging between 1.25×10^{-6} to 1.52×10^{-10}M.

Table S1

bsFpF 2	Ligand	k_a (1/Ms) × 10^4	k_d (1/s) × 10^{-4}	K_D (nM)
Fab_{VEGF}-PEG_{15}-Fab_{IL6R}	IL6R	2.80	5.71	20.3
Fab_{VEGF}-PEG_{15}-Fab_{IL6R}	VEGF	0.84	1.0	12.0
$Fab_{TNF\alpha}$-PEG_{15}-Fab_{IL6R}	TNF_{α}	8.81	5.34	5.9
$Fab_{TNF\alpha}$-PEG_{15}-Fab_{IL6R}	IL6R	9.15	2.63	2.9
Fab_{VEGF}-PEG_{15}-$Fab_{TNF\alpha}$	VEGF	0.81	0.81	10.0
Fab_{VEGF}-PEG_{15}-$Fab_{TNF\alpha}$	TNF_{α}	7.03	0.84	2.4

Table S1. Average kinetic constant rates of the bsFpFs using conjugation-ligation reagents **28** and **30** measured by SPR techniques

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