Electronic Supplementary Information

Fc-fusion mimetics

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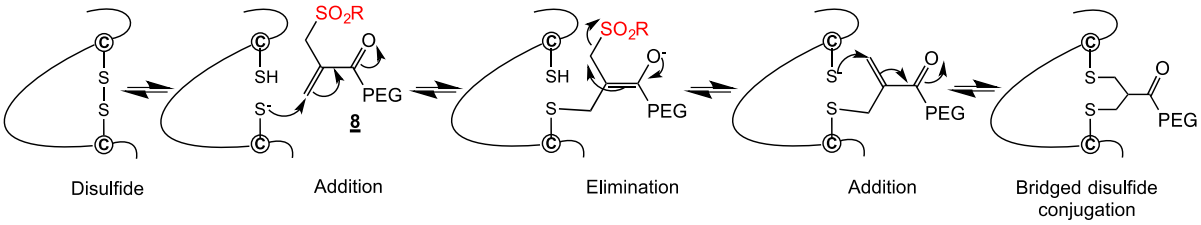
Disulfide bridging PEG conjugation.

The use of *bis*-alkylation for conjugation can be utilised for the site-specific conjugation of the two cysteine thiols derived from a native disulfide.[1] Conjugation is thought to occur by an addition-elimination pathway shown in Scheme S1. This approach to site-specific conjugation was initially developed for site-specific PEGylation and has now been shown to be a valuable approach for the development of antibody drug conjugates[2] as well as the fabrication of FpFs[3] and now RpRs. PEG mono-sulfone reagents **8** have also been shown to undergo site-specific conjugation to histidine tags.[4]



Scheme S1. The *mono*-sulfones **8** are latently crossed functionalised reagents capable of sequential and interactive addition-elimination reactions capable of bis-alkylation. In the case of disulfides, first the cysteine thiols are liberated by reduction (e.g. TCEP[1b] or DTT) and then conjugation involves (i) a first thiol addition to the mono-sulfone reagent **8**, (ii) sulphinic acid elimination to generate a second double bond, and (iii) a second thiol addition.

Experimental details

Preparation of sodium phosphate buffer (20 mM, pH 7.4) with EDTA (10 mM)

To a 500 mL Fisher bottle with a magnetic stir bar was added sodium phosphate monobasic, NaH_2PO_4 (600 mg, Mw=119.98 g/mol, 5 moles), EDTA (931 mg Mw = 372.24 g/mol, 2.5 mmole) and distilled water (250 mL, Type 1, 18 mΩ resistance). The solution was gently stirred until homogeneous and using a pH meter, the pH was carefully adjusted to 7.4 by the dropwise addition of sodium hydroxide (1 N).

*Dithiothreitol reduction of aflibercept to give $VEGFR_1$-$VEGFR_2$-Fc **5***

Into an eppendorf (1.5 mL) was added dithiothreitol (DTT, 1.0 mg) and a solution of aflibercept (1.0 mg, 1.0 mL in the sodium phosphate buffer (20 mM, pH 7.6) with EDTA (10 mM). This solution was allowed to incubate at ambient temperature without shaking for 30 min. The monomer $VEGFR_1$-$VEGFR_2$-Fc fragment **5** was obtained after removal of DTT by PD-10 column by buffer exchanging into a sodium phosphate buffer (20 mM, pH 7.6) with EDTA (10 mM). A representative procedure to remove the DTT from a volume of 1.0 mL of reaction mixture using a PD-10 column was as follows: first a PD-10 column was equilibrated by allowing solutions of sodium phosphate buffer (20 mM, pH 7.6, 25 mL) with EDTA (10 mM) to elute through the column. Then, the protein solution with DTT (1.0 mL) was loaded onto the equilibrated PD-10 column. After this, 1.5 mL sodium phosphate buffer (20 mM, pH 7.6) with EDTA (10 mM) was added to the PD-10 column and allowed to elute. Finally, the protein solution was recovered by addition of a 3.3 mL sodium phosphate buffer (20 mM, pH 7.6) with EDTA (10 mM) to the PD-10 column.

Ellman's reagent (4.0 mg, DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid; Thermo Fisher, cat. no. 225820) was dissolved in 1.0 mL of the reaction buffer (sodium phosphate buffer (20 mM), EDTA (1.0 mM), pH 8.0). Cysteine standard solutions in the reaction buffer were prepared using cysteine hydrochloride monohydrate at the concentrations of 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mM. A blank solution with no cysteine hydrochloride was also prepared. An aliquot of each standard concentration (250 µL) and blank was added to a solution comprised of the Ellmans reagent (50 µL) and the reaction buffer (2.5 mL). These mixtures were allowed to incubate for 15 min at ambient temperature and then the absorbance of each solution was measured (412 nm) relative to blank.

The concentration of thiols in the sample solution (0.2 mL) was first calculated. Then, the value was divided by the concentration of protein in the sample solution to obtain the number of thiols in the solution. No free thiol was observed with native aflibercept. However, monomeric $VEGFR_1$-$VEGFR_2$-Fc fragment **5** (4.5×10^{-3} mM) after DTT treatment displayed

an absorbance of 0.054 at 412 nm. This was equal to 4 free thiols for the starting aflibercept indicating the presence of two interchain disulfide bonds.

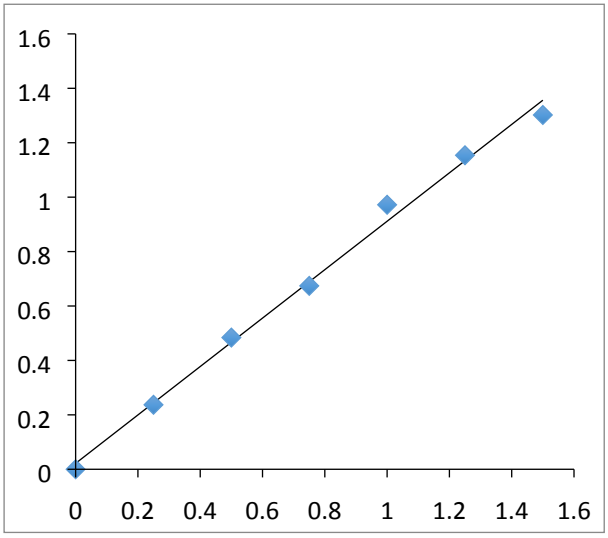


Figure 1S. The calibration curve for cysteine standard solutions at 412 nm for the Ellmans assay.

Samples	Absorbance
Cysteine 0.0 mM (blank)	0.005
Cysteine 0.25 mM	0.263
Cysteine 0.5 mM	0.51
Cysteine 0.75 mM	0.699
Cysteine 1.0 mM	0.998
Cysteine 1.25 mM	1.18
Cysteine 1.5 mM	1.32
Aflibercept (9×10^{-3} mM)	0.031
Reduced-aflibercept (4.5×10^{-3} mM)	0.054

Table 1S. Absorption at 412 nm for aflibercept before and after DTT reduction in the presence of Ellman's reagent.

*Proteolytic digestion of aflibercept to prepare the dimeric $VEGFR_1$-$VEGFR_2$ fragment **6***

Immobilised IdeS enzyme (FabRICATOR®, FragIT MidiSpin, Genovis; Cat no A0-FR6-100) was used for the proteolytic digestion of aflibercept. The top lid and the bottom cap of the FragIT MidiSpin column was removed and the column centrifuged at 100 × g for 1.0 min to remove storage buffer. The column was then equilibrated with cleavage buffer (2.5 mL; 50 mM sodium phosphate, 150 mM NaCl, pH 6.6) and centrifuged twice at 100 × g for 1 min. The bottom of the column was then capped and secured with parafilm to stop any leakage. Aflibercept (6 mg in 1.0 mL cleavage buffer) was then added to the column and the top lid was sealed. The aflibercept digestion solution was incubated on the column for 30 min at ambient temperature by end-over-end mixing. After 30 min, the top lid and bottom cap were removed and spin column was centrifuged at 100 × g for 1.0 min. The column was then washed with cleavage buffer (1.0 mL) and centrifuged at 100 × g for 1.0 min.

The digestion mixture and washing solutions were combined. Analysis by SDS-PAGE confirmed the presence of the $VEGFR_1$-$VEGFR_2$ dimer **6**, which was then purified by

elution over a CaptureSelect MidiSpin column (Genovis, Cat no A2-FR2-100). The column is packed with a multi species Fc affinity matrix on agarose beads that binds Fc containing fragments from solution. The CaptureSelect column was first pre-equilibrated with binding buffer (3.0 mL; 10mM sodium phosphate, 150mM NaCl, pH 7.4) and then centrifuged at 200 × g for 1.0 min to remove the binding buffer. The pooled aflibercept digestion solution was then added to the CaptureSelect column, which was then sealed and the column was allowed to incubate for 30 min at ambient temperature with end-over-end mixing. The column was then centrifuged at 200 × g for 1 min and the first fraction containing the purified $VEGFR_1$-$VEGFR_2$ dimer **6** was obtained. The column was then washed with binding buffer (1.0 mL) and then centrifuged (200 × g, 1.0 min) two times to ensure as much $VEGFR_1$-$VEGFR_2$ dimer **6** could be recovered as possible. The solutions containing the purified $VEGFR_1$-$VEGFR_2$ dimer **6** were then combined and analysed by SDS-PAGE and the concentration was determined using a micro BCA assay. From 6 mg of aflibercept, it was possible to obtain 2 mg of $VEGFR_1$-$VEGFR_2$ dimer **6**. Removal of Fc containing species from the CaptureSelect column was achieved using glycine buffer (pH 1.5).

*Preparation of RpR **2** general procedure*

Dimeric $VEGFR_1$-$VEGFR_2$ fragment **6** (0.8 mg/mL, 1.0 mL PBS pH 7.3) was treated with DDT (1.0 mg, 6.0 mM) without shaking for a 30 min period at ambient temperature to give $VEGFR_1$-$VEGFR_2$ monomer **4**. The DDT reaction mixture was buffer exchanged (sodium phosphate buffer (20 mM), EDTA (10 mM) and pH 7.6) to remove the DDT using a PD-10 column. To a solution of monomeric $VEGFR_1$-$VEGFR_2$ **4** (3.3 mL, 0.24 mg/mL) was added 0.9 equivalents the PEG di(mono-sulfone) reagent **3** (0.12 mg PEG reagent **3**, 10 kDa) and the reaction mixture was incubated for 12 h at 4 ^{0}C without shaking. The reagent **3** was prepared as previously described.[3] RpR **2** was then purified from the reaction mixture by size exclusion chromatography (SEC), which was conducted using a superpose 12 HR 10/30 size exclusion column (30 µm particle size) and phosphate buffered saline (PBS) solution (NaCl (0.16 M), KCl (0.003M), Na_2HPO_4 (0.008M) and KH_2PO_4 (0.001M) at pH 7.3). A run time of 40 min using a flow rate of 1.0 mL/min were applied. Fractions (1.0 mL) were collected and analysed using SDS-PAGE (Figure 2S, lanes 2-10). The SEC fractions containing the desired RpR **2** (Figure 2S, lanes 4-6) were pooled and concentrated to 1.0 mL solution using VIVA-SPIN. The concentration of RpR **2** was then determined by by micro-BCA assay. From 0.8 mg of starting $VEGFR_1$-$VEGFR_2$ dimer **6**, 0.2 mg of purified RpR fragment **2** was obtained.

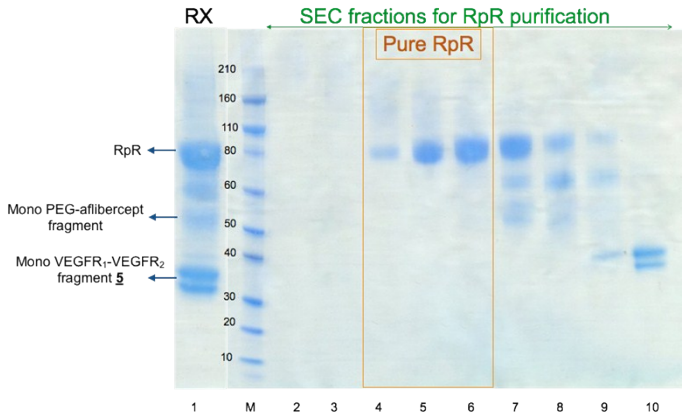


Figure 2S. SDS-PAGE gels of reaction mixture between $VEGFR_1$-$VEGFR_2$ dimer **6** (1.0 mg/mL) and PEG reagent **3** (1 eq, 10 kDa), purification of RpR using size-exculsion chromatography (SEC); Novex Bis-Tris 4-12% gel stained with colloidal blue for protein. *M:* standard protein markers, *Lane 1:* Conjugation reaction mixture, *Lanes 2-10*: SEC fractions for RpR purification.

*Thiol-capped $VEGFR_1$-$VEGFR_2$ fragment **7***

Iodoacetimide (18 mM, 5.5 mg) was added to a solution of $VEGFR_1$-$VEGFR_2$ monomer **4** (0.35 mg, 1.65 mL). After incubation for 1.0 h at ambient temperature in the dark, the solution was then buffer exchanged to the sodium phosphate buffer (20 mM), EDTA (10 mM) and pH 7.6 using a PD-10 column to remove iodoacetamide species.

*Determination of the solution size of RpR **2** by static light scattering*

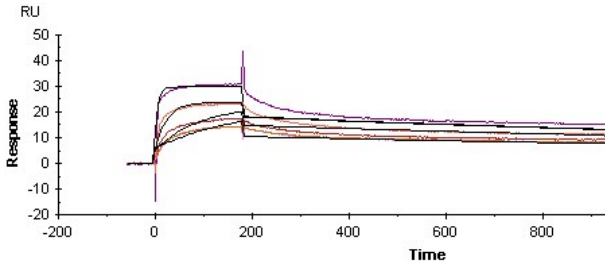
The DynoPro plate reader II (Wyatt technology) measures the size (hydrodynamic radius) and size distribution of the protein from a 0.5 nm to 1000 nm range using a dynamic light scattering (DLS) method. Samples (aflibercept, $VEGFR_1$-$VEGFR_2$ dimer **6**, thiol capped $VEGFR_1$-$VEGFR_2$ fragment **7** and RpR **2**) were prepared at concentration of 0.3 mg/mL using PBS buffer which was passed through a 0.2 µm filter. Into each well of the 384 well plate (Corning, cat no 23714026), 30 µL of sample was added (three replicates for each sample) and analysed at 25 ^{0}C.

*Binding properties of RpR **2** determined by surface plasmon resonance*

BIAcore X-100 (BIAcore, GE healthcare LTD Amersham) was used for binding affinity studies of the aflibercept, $VEGFR_1$-$VEGFR_2$ dimer **6** and RpR **2**. Human recombinant $VEGF_{165}$ (38 kDa Mw) was immobilised on CM3 chip at an immobilisation level of 91 RU using standard carbodiimide-mediated coupling (NHS/EDC, 50/50) and ethanolamine (pH 8.5). Samples were prepared in HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, 0.005% surfactant P20). All kinetic measurements were conducted at 25 ^{0}C at a flow rate of 30 µL/min with an association time of 180 s and dissociation time of 1200 s. Chip regeneration was accomplished by exposure to 10.0 mM glycine−HCl (pH 2.0) for 30 s. Double-referencing was performed to account for bulk effects caused by changes in the buffer composition or nonspecific binding. Data were evaluated with BIAevaluation software (version 2.1) and the best fit (lowest χ^2) was obtained using a 1:1 binding model. The sensorgram was fitted globally over the association and dissociation phases.

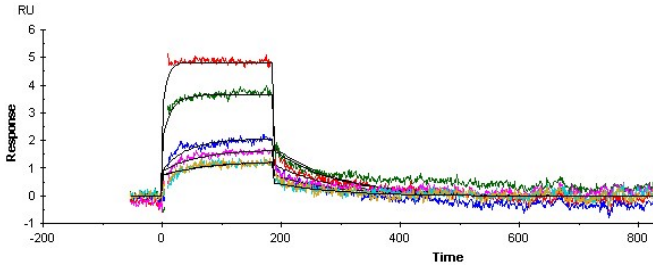
Equilibrium dissociation constants (affinity) were calculated from the rate constants ($K_D = k_d / k_a$). Figure 3S show representative fitting curves for aflibercept and RpR using 1:1 binding model.

(A) Aflibercept



tc	Chi² (RU²)	U-value
3.34 x 10^{15}	0.823	5

(B) RpR 2



tc	Chi² (RU²)	U-value
1.59 x 10^{11}	0.116	12

Figure S3. Representative fitting curves for aflibercept and RpR **2**.

There are parameters such as the tc, Chi² and U-value which help to assess the fitting model. The Chi² is the sum of the squared error between the fitted curve and experimental curve. This number should be as small as possible (less than 2 and even less than 1). The parameter which represents the effect of mass transport limitation is tc. If the tc value is between 10^7 to 10^9, then there is a mass transport limitation. The U-value is a parameter that represents the uniqueness of the calculated rate constant and R_{max}. Lower U-values indicate greater confidence in the results. A high value (above 25) indicates that the reported kinetic rate constants are not useful.

The binding chart (Figure S4) for $VEGFR_1$-$VEGFR_2$ dimer **6** and $VEGFR_1$-$VEGFR_2$ monomer **7** illustrates the increased binding in aflibercept due to bivalency.

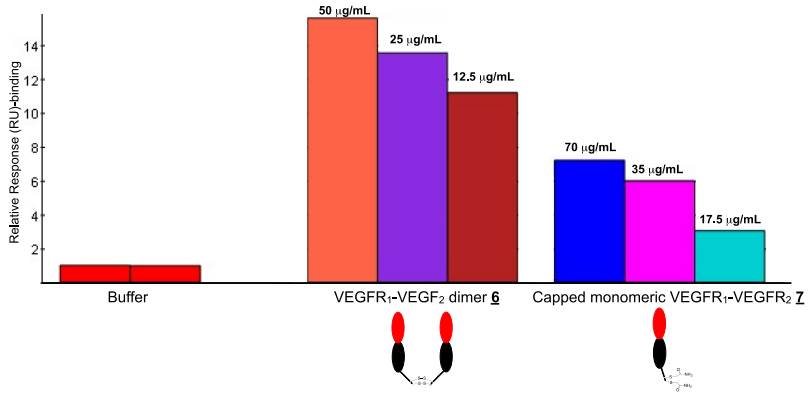


Figure S4. Binding chart for $VEGFR_1$-$VEGFR_2$ dimer **6** and thiol-capped monomeric $VEGFR_1$-$VEGFR_2$ **7**.

*Functional activity of RpR **2** using in vitro anti-angiogenesis co-culture assay*

A HUVEC co-culture assay (V2a kit, cat no ZHA-4000, TCS Cellworks Ltd.) was used according to the manufacturer' s instructions. Test samples were sterilized using filter (0.22 µm) and diluted in growth medium to their final required concentrations and added to each well (0.5 mL per well) of 24-well plate. The concentration of $VEGF_{165}$ used was 10 ng/mL in each well and the concentrations of the RpR **2** were normalized for their protein molecular weights. For example, for molar ratios of 3:1, 1.5:1, and 0.5:1 of sample to VEGF, in well concentrations of 0.06, 0.03, and 0.01 µg/mL of RpR **2**, and 0.08, 0.04 and 0.015 µg/mL of aflibercept were required. The samples were pre-incubated with $hVEGF_{165}$ for 2 h at 37 ^{0}C before addition to the cells. As negative control, designated wells were treated with medium only (no VEGF) and for positive controls treated with only VEGF (10 ng/mL) and VEGF (10 ng/mL) with anti-mouse anti-TNFα IgG (0.08 µg/mL). Triplicate wells were prepared for each test environment. The assay plate was then placed in a humidified incubator (37 ^{0}C, 5% CO_2). Media was replaced with fresh culture media containing the test samples on days 4, 7, and 9. On day 10, cells were fixed (ice-cold 70% ethanol; 0.5 mL per well). Cells were first exposed to mouse anti-human CD31 primary antibody (1:400 dilution, 0.5 mL per well, 60 min at 37 ^{0}C), followed by alkaline phosphatase-linked goat anti-mouse secondary antibody (1:500 dilution, 0.5 mL per well, 60 min at 37 ^{0}C). Cells were then rinsed and permanently stained for CD31 using insoluble 5-bromo-4-chloro-3-indolylphosphate/nitroblue-tetrazolium salt. Plates were air-dried and photomicrographs were taken using an upright microscope. The images were subsequently analyzed using AngioSysn (AngioSys Image Analysis Software, TCS Cellworks Ltd) software to calculate the number of junctions and tubules (Figure S5) formed in each well.

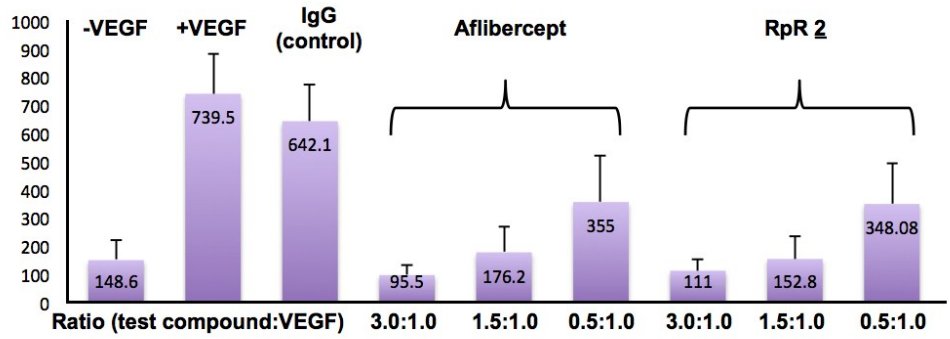


Figure S5. Tubule formation observed for medium alone, medium + VEGF, Anti-mouse TNF-a IgG + VEGF, aflibercept + VEGF and RpR + VEGF. Ratios are the amount of test compound to VEGF. VEGF was present at a fixed concentration of 10 ng/mL.

References

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