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Fc-fusion mimetics

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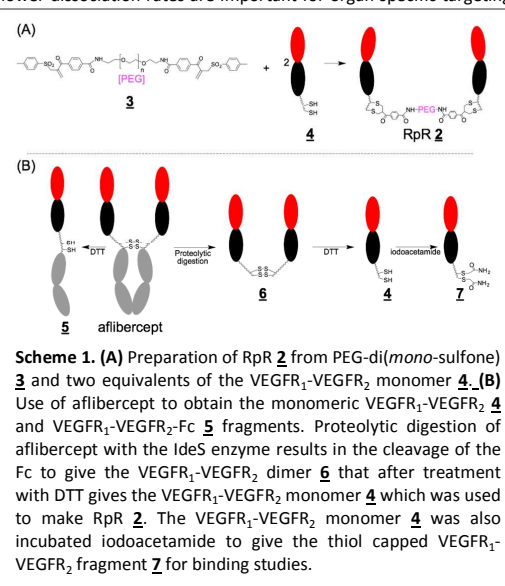
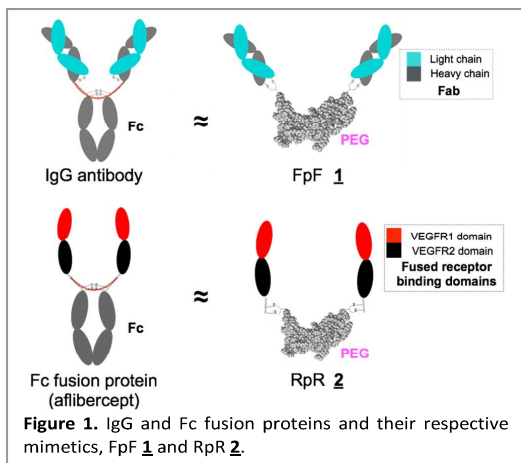
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The Fc-fusion mimetic RpR **2** was prepared by disulfide bridging conjugation using PEG in the place of the Fc. RpR **2** displayed higher affinity for VEGF than aflibercept. This is caused primarily by a slower dissociation rate, which can prolong a drug at its site of action. RpRs have considerable potential for development as stable, organ specific therapeutics.

To achieve effective bivalency and high affinity, the two Fabs in an IgG antibody are mobile and are linked together as if each Fab (or protein) is bound at the end of linear molecule (Figure 1). FpFs **1** are IgG antibody mimetics (Figure 1) designed to have enhanced stability and binding properties compared to IgGs. They are prepared from PEG-di(mono-sulfone) **3** and two antibody fragments (Fabs).¹ Fc-based fusion proteins² (Figure 1) are also capable of

exploiting the therapeutic advantages of bivalency that are displayed by IgGs. Several Fc-fusion proteins are registered for clinical use³ and they will continue to offer considerable clinical potential because of Fc recycling, but they can be difficult to produce during early preclinical research and to scale for production.⁴ Fc-fusion proteins are also often prone to aggregation during downstream processing⁵ and have similar stability limitations as IgGs. There are therapeutic applications where the Fc is not needed or can cause problems.⁶ One area of interest is the use of antibody based medicines in organ specific applications such as the eye. In such cases, Fc recycling does not occur and effector function can be deleterious, especially in the treatment of inflammatory conditions. Improved stability is important to formulate more concentrated solutions to decrease the frequency of dose administration and improved binding properties such as slower dissociation rates are important for organ specific targeting.



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In an effort to further explore the potential of antibody-based mimetics that are made using the PEG-di(*mono*-sulfone) **3** (Scheme 1A, Figure 1) we describe an Fc-fusion mimetic that we call RpR **2**, for receptor binding region-PEG-receptor binding region.

Aflibercept (Eylea) is a Fc-fusion protein that is used to treat age related macular degeneration (AMD) by binding to vascular endothelial growth factor (VEGF) in the back of the eye. It is administered by intravitreal injection directly into the eye. Fc-fusion proteins are related to IgG antibodies in that both have an Fc domain. IgG antibodies have two heavy and two light chains. The Fc-domain comprises the C2 and C3 regions of the two heavy chains. Disulfide bonds exist in IgG antibodies that are between the heavy chains in the hinge region to separate the Fc domain from the hinge and Fabs. Aflibercept is a homodimer that comprises 2 monomers of (VEGFR₁-VEGFR₂)-(C2-C3), referred to here as VEGFR₁-VEGFR₂-Fc **5** (Scheme 1B), with disulfide bonds expected to be in the peptide sequence between the target binding domains (VEGFR₁-VEGFR₂) and the Fc domain (C2-C3 regions) (Figure 1).⁷ Aflibercept is glycosylated with a total molecular weight of 115 kDa, of which 97 kDa is due to the protein component. Each monosulfone moiety in reagent **3** undergoes site-specific conjugation with the two cysteine thiols from a disulfide bond by a sequence of addition-elimination reactions to insert a stable 3-carbon methylene bridge between the two thiols of the original disulfide bond (Scheme S1, ESI).⁸ The thiol ether bonds in a rebridged disulfide bond are more stable than the original disulfide bond. To make the desired RpR **2** we first had to obtain the VEGFR₁-VEGFR₂ fragment **4** by proteolytic digestion of aflibercept to remove the Fc domain (Scheme 1B).

It was first confirmed that aflibercept migrated to an approximate molecular weight of about 115 kDa by SDS PAGE (Figure 2, lane 1). Aflibercept was then treated with dithiothreitol (DTT) to reduce the accessible disulfide bonds thought to exist in a hinge like region between the binding domain (VEGFR₁-VEGFR₂) and the Fc domain. A broad band appeared at ~55-60 kDa by SDS-PAGE (Figure 2, lane 2). We believe this band corresponds to the monomeric VEGFR₁-VEGFR₂-Fc **5** (Scheme 1B). Glycosylation is usually somewhat heterogeneous in therapeutic proteins, so we inferred that the broadness of the band at ~55-60 kDa was due to glycosylation heterogeneity. DTT was then removed using a PD-10 column and the reduced aflibercept solution was incubated with Ellman's reagent which indicated the presence of 4 accessible cysteine thiols in aflibercept (Figure 1S, Table 1S, ESI). This suggested that there are 2 cysteines in each VEGFR₁-VEGFR₂-Fc **5** monomer which can form two disulfide bonds in aflibercept analogous to what is found in the hinge region of IgG antibodies. Hence it was thought possible that an RpR **2** derived from aflibercept could be prepared using the PEG-di(*mono*-sulfone) **3**. If only one cysteine had been present in the VEGFR₁-VEGFR₂-Fc **5** monomer, there are stable, mono-thiol conjugation linkers available⁹ that would have been utilised in a bifunctional reagent analogous to PEG-di(*mono*-sulfone) **3**.

Proteolytic digestion of aflibercept was then examined in an effort to obtain the monomeric VEGFR₁-VEGFR₂ fragment **4** (Figure 3). Preliminary digestion studies of aflibercept using immobilised papain yielded only difficult to characterise small peptide fragments. We had previously used papain to digest IgGs to obtain

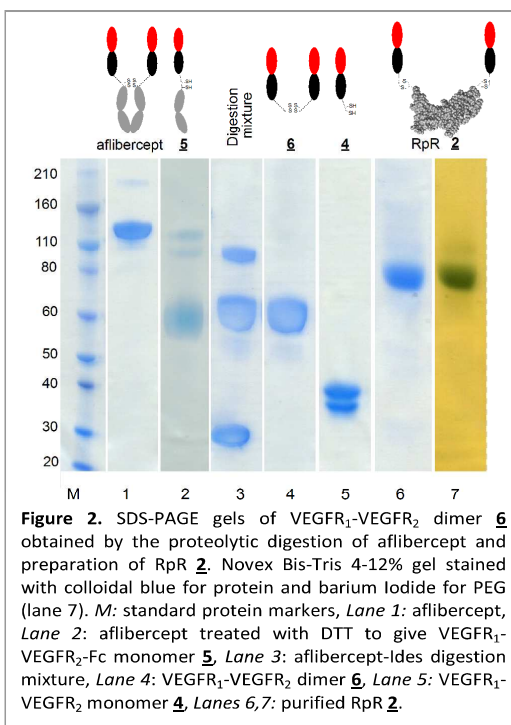


Figure 2. SDS-PAGE gels of VEGFR₁-VEGFR₂ dimer **6** obtained by the proteolytic digestion of aflibercept and preparation of RpR **2**. Novex Bis-Tris 4-12% gel stained with colloidal blue for protein and barium iodide for PEG (lane 7). *M*: standard protein markers, *Lane 1*: aflibercept, *Lane 2*: aflibercept treated with DTT to give VEGFR₁-VEGFR₂-Fc monomer **5**, *Lane 3*: aflibercept-IdeS digestion mixture, *Lane 4*: VEGFR₁-VEGFR₂ dimer **6**, *Lane 5*: VEGFR₁-VEGFR₂ monomer **4**, *Lanes 6,7*: purified RpR **2**.

Fabs,^{1, 10} but recognised that proteolytic digestion of different antibody subclasses and motifs can be difficult to control.¹¹

A second proteolytic enzyme (IdeS enzyme, FabRICATOR[®], Genovis) that can cleave an IgG at glycine-glycine bonds¹² in the hinge reagent to give F(ab)₂ was then examined. Incubation of aflibercept with IdeS resulted in 3 bands by SDS-PAGE (Figure 2, lane 3). New bands appeared at approximately 30 kDa and 60-70 kDa. A third band at approximately 95-100 kDa in this gel was thought to be undigested aflibercept. The lower molecular weight fragment at 30 kDa, which is thought to be the cleaved Fc, and the band for undigested aflibercept were removed by eluting the digestion mixture over a column that binds to the Fc domain (CaptureSelect Midispin, Genovis). This provided a purified a non-Fc containing fragment at 60-70 kDa (Figure 2, lane 4) which was thought to be the VEGFR₁-VEGFR₂ dimer **6** (Scheme 1B).

Incubation of VEGFR₁-VEGFR₂ dimer **6** with DTT caused this fragment to disappear to give 2 lower molecular weight fragments (Figure 2, lane 5). These fragments are thought to be the desired VEGFR₁-VEGFR₂ monomer **4** (Scheme 1). Two bands are often observed after reduction of Fabs that are obtained by proteolytic digestion. This can be due to miscleavage reactions during proteolysis and may be exacerbated for aflibercept due to its difference in structure compared to an IgG and due to aflibercept glycosylation. There are five N-glycosylation sites on each monomeric VEGFR₁-VEGFR₂-Fc fragment **5** which may be partially or completely glycosylated. There may also be additional heterogeneity caused by differences in saccharide structure.

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To prepare the RpR **2**, the VEGFR₁-VEGFR₂ dimer **6** was first incubated with DTT for 30 minutes to give the VEGFR₁-VEGFR₂ monomer **4** (Scheme 1B). The reaction mixture was carefully eluted over a PD-10 column to remove the DTT while avoiding disulfide reformation, and then the PEG di(mono-sulfone) reagent **3** (derived from a 10 kDa PEG precursor) was added to the solution of the monomeric VEGFR₁-VEGFR₂ **4**. Incubation of the reaction mixture for 3 h (Figure 2S, lane 1, ESI) was then followed by purification by size exclusion chromatography (Figure 2S, lanes 2-10, ESI) to give the purified RpR **2** which appeared in a band at approximately 70 kDa (Figure 2, lanes 6 and 7). Two detection dyes were used, first coomassie blue to detect protein (lane 6) and then barium iodide to detect the PEG (lane 7) being conjugated to the protein. Starting from 0.8 mg (in 1.0 mL) of VEGFR₁-VEGFR₂ dimer **6**, approximately 0.16 mg (in 0.5 mL) of RpR **2** was obtained (~ 20 % yield).

At 25 °C the purified RpR **2** displayed a solution size of 10.7 ± 0.5 nm (Pd, 0.7 ± 0.1 nm), which is similar to the starting aflibercept (10.2 ± 0.7 nm; Pd, 0.6 ± 0.1 nm). The FpF antibody mimetics **1** were also a similar solution size to the corresponding IgG.¹ This is in stark contrast to when PEG is conjugated only at one terminus to a single protein where the solution size of a PEG-protein conjugate is dominated by the random coil nature of PEG.¹³ When only one terminus of PEG is conjugated to a protein, the other PEG terminus has considerable freedom to allow the PEG to maintain a large solution structure.

As a macromolecule, the RpR has a protein at each terminus of the PEG scaffold, which ~~would constrain the motion of the PEG, so this Fc-fusion mimetic~~ is analogous to an A-B-A block copolymer where it is known that ~~the two functionalised end blocks linear polymers functionalised at both ends~~ can self-associate.¹⁴ The dimeric VEGFR₁-VEGFR₂ fragment **6** also displayed a solution size of 10.03 ± 0.1 nm (Pd, 0.7 ± 0.1 nm) which is similar to both aflibercept and RpR **2**. Interestingly, when the VEGFR₁-VEGFR₂ fragment **6** was treated with DTT and the cysteine thiols were blocked with iodoacetamide, the cysteine thiol-capped monomeric VEGFR₁-VEGFR₂ fragment **7** (Scheme 1B) displayed a solution size of 7.2 ± 0.4 nm (Pd, 0.7 ± 0.1 nm). Although the dimer **6** is twice the molecular weight of the VEGFR₁-VEGFR₂ monomer **7**, ~~its solution size is only about 40% larger~~ suggesting that there ~~may be some non-covalent~~ intramolecular association ~~of the between each of the VEGFR₁-VEGFR₂ domains monomers~~ within the dimer **6**.

The binding properties of the RpR **2** and aflibercept were then evaluated by surface plasmon resonance (Biacore) to determine the affinity (K_D), and the rate constants of association (k_a) and dissociation (k_d) (Table 1). Vascular endothelial growth factor-165 (VEGF₁₆₅), which is a ligand for aflibercept, was immobilised at a density to minimise or prevent rebinding events (91 RU).^{1, 15} The dissociation rate (k_d) for the RpR **2** was slower than what was observed with aflibercept. Interestingly, the k_a appeared to be slightly faster in RpR **2** compared to aflibercept. This is in contrast to what was previously observed for anti-VEGF FpF which had a slower association rate than the precursor IgG antibody.¹ However it was the decreased k_d of RpR **2** that appeared to be the

dominating factor to cause the improved affinity of RpR **2** compared to aflibercept (Table 1). Representative fitting curves for aflibercept and RpR **2** are shown in the ESI (Figure S3, ESI).

Exploiting reduced dissociation rates may be a viable strategy to increase efficacy by increasing the residence time and mode of action within specific tissue.¹⁶ Although the reduction in k_d for FpF **1** is also slower than the parent IgG,¹ there appears to be a greater relative reduction in k_d for the RpR **2** compared to its parent Fc-fusion (i.e. aflibercept). During initial dissociation steps from the ligand of one of the two VEGFR₁-VEGFR₂ domains in the RpR **2**, PEG conformational flexibility may be more efficient for rebinding than the polypeptide linking the Fc domain to the VEGFR₁-VEGFR₂ domain in aflibercept. This suggests there is less flexibility in the bivalent binding moieties in the Fc-fusion protein (aflibercept) than there is in an IgG (e.g. bevacizumab).

The VEGF binding of the capped VEGFR₁-VEGFR₂ monomer **7** (Figure S4, ESI) was reduced when compared to the VEGFR₁-VEGFR₂ dimer **6**. This exemplified the advantages of the cooperative bivalent binding that is possible with (i) aflibercept, (ii) the dimeric VEGFR₁-VEGFR₂ fragment **6** and (iii) RpR **2** (Table 1). The similar binding properties that were observed for both the dimer **6** and aflibercept suggests that the placement of the accessible disulfide bonds linking each monomer in aflibercept is important for the mobility of the VEGFR₁-VEGFR₂ binding domains. Inclusion of a polypeptide sequence to extend the VEGFR₁-VEGFR₂ receptor domains away from the aflibercept disulfide bonds to better optimise dissociation rates would be expected to make aflibercept less stable. Such an added polypeptide sequence to increase the flexibility of the VEGFR₁-VEGFR₂ receptor domains would invariably lack secondary structure in a similar way to the hinge region of IgG antibodies. While the hinge region in IgG antibodies provides the flexibility needed for cooperative and bivalent binding of both Fabs,¹⁷ the IgG hinge region is also vulnerable to degradation and disulfide scrambling.¹⁸ The stable conjugation imparted by PEG-di(mono-sulfone) **3** and use of a PEG scaffold provides enough flexibility of the VEGFR₁-VEGFR₂ binding moieties to potentially maximise both association and dissociation rates that could be important in the development of new therapeutics.

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Sample	k_a ($\times 10^5$) $M^{-1}s^{-1}$	k_d ($\times 10^{-4}$) s^{-1}	K_D (k_d/k_a) nM
Aflibercept	0.88	4.20	4.78
(VEGFR ₁ -VEGFR ₂) ₂ 6	1.20	5.20	4.30
RpR 2	1.13	1.90	1.71

Table 1. Binding kinetic constant rate of aflibercept, VEGFR₁-VEGFR₂ dimer **6** and RpR **2**, Affinity, dissociation and association rate constants were studied using CM3 chip immobilised with 91 RU human VEGF at 25 °C. The range of concentrations used for the conjugates was 0.06 μM to 1.6 μM. Data were collected from 2 independent experiments for RpR **2** and 3 independent experiments for aflibercept and VEGFR₁-VEGFR₂ dimer **6**.

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RpR **2** was then evaluated *in vitro* using a human umbilical vein endothelial cell (HUVEC) co-culture (Figure 3). This assay measures the migration and the formation of an anastomosing network that is characterised by tubule and junction formation during HUVEC proliferation. These processes are characteristic for angiogenesis and are often a good *in vitro* measurement for angiogenesis.¹⁹ RpR **2** and aflibercept were incubated with VEGF₁₆₅ at different molar ratios of 3.0, 1.5, 0.5 for 2 hours at 37°C prior to incubation with HUVECs. VEGF₁₆₅ and anti-mouse TNF- α IgG were used for positive controls. Images were obtained after fixing HUVECs with an anti-CD31 antibody to differentiate between the endothelial tubular network and non-endothelial structures of similar apparent morphology (Figure 3A). These images suggest that both aflibercept and RpR **2** have similar anti-angiogenic properties. Quantification of tubule (Figure S5, ESI) and junction formation (Figure 3B) (AngioSys Image Analysis Software, TCS Cellworks Ltd.) showed that the formation of these structures were similarly inhibited in a concentration dependent manner by both RpR **2** and aflibercept.

To summarise, a new antibody Fc-fusion mimetic called an RpR was prepared. Aflibercept is a clinically used Fc-fusion protein that targets VEGF was used for these studies. Proteolytic digestion of aflibercept followed by incubation with DTT provided the monomeric VEGFR₁-VEGFR₂ domain **4** that was then conjugated to the PEG-di(mono-sulfone) **3** by disulfide bridging conjugation to

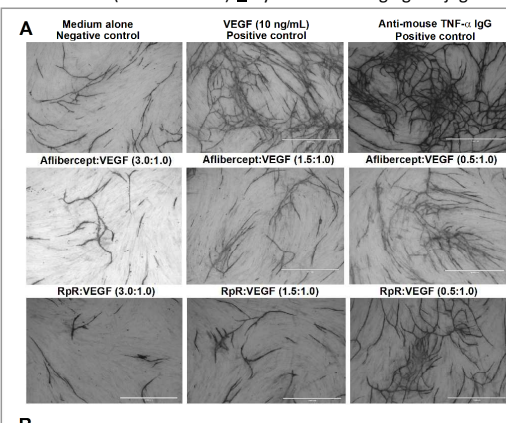


Figure 3. (A) Representative images that were used for AngioSys analysis to quantitate tubule formation using a HUVEC-fibroblast angiogenesis assay. The dark structures (tubules) are indicative of angiogenesis and were analysed to determine the number of junctions. (B) Number of junctions observed for medium alone, medium + VEGF, anti-mouse TNF- α 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. Data are expressed as the mean of three individual cultures per treatment environment.

give the anti-VEGF RpR **2**. The strategy to proteolytically digest aflibercept provided the means to compare the properties of the RpR Fc-fusion mimetic **2** with aflibercept. The solution size of RpR **2** and its *in vitro* activity are comparable to aflibercept. Of most interest is that binding studies show that RpR **2** has higher affinity for VEGF compared to aflibercept primarily due to a slower dissociation rate. Antibody based mimetics such as RpR **2** have potential for development as stable, organ specific therapeutics.

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Electronic Supplementary Information

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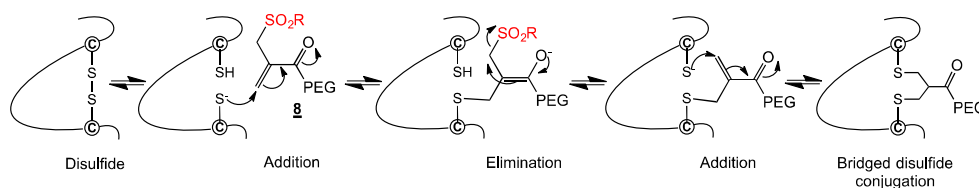
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Disulfide bridging PEG conjugation.

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 conjugates² as well as the fabrication of FpFs³ 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) sulphenic 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, $M_w=119.98$ g/mol, 5 moles), EDTA (931 mg $M_w = 372.24$ g/mol, 2.5 mmole) and distilled water (250 mL, Type 1, 18 m Ω 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 VEGFR₁-VEGFR₂-Fc 5

~~"Dithiothreitol"~~ with ~~"Dithiothreitol"~~ 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 VEGFR₁-VEGFR₂-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 VEGFR₁-VEGFR₂-Fc fragment 5 (4.5×10^{-3} mM) after DTT treatment displayed

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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.

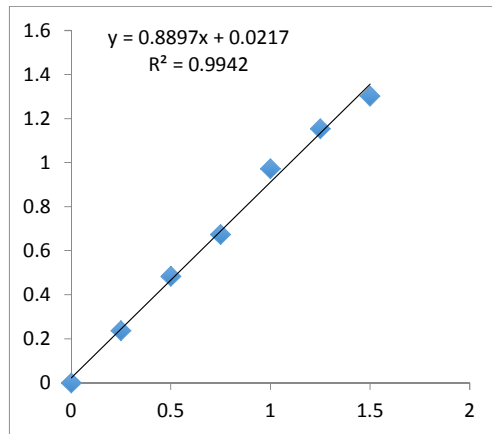


Figure 1S. The calibration curve for cysteine standard solutions at 412 nm for the Ellmans assay.

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 VEGFR₁-VEGFR₂ 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 $100 \times g$ 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 $100 \times g$ 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 $100 \times g$ for 1.0 min. The column was then washed with cleavage buffer (1.0 mL) and centrifuged at $100 \times g$ for 1.0 min.

The digestion mixture and washing solutions were combined. Analysis by SDS-PAGE confirmed the presence of the VEGFR₁-VEGFR₂ 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 $200 \times g$ 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 $200 \times g$ for 1 min and the first fraction containing the purified VEGFR₁-VEGFR₂ dimer **6** was obtained. The column was then washed with binding buffer (1.0 mL) and then centrifuged ($200 \times g$, 1.0 min) two times to ensure as much VEGFR₁-VEGFR₂ dimer **6** could be recovered as possible. The solutions containing the purified VEGFR₁-VEGFR₂ 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 VEGFR₁-VEGFR₂ 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 VEGFR₁-VEGFR₂ 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 VEGFR₁-VEGFR₂ 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 VEGFR₁-VEGFR₂ **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 °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), Na₂HPO₄ (0.008M) and KH₂PO₄ (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 micro-BCA assay. From 0.8 mg of starting VEGFR₁-VEGFR₂ dimer **6**, 0.2 mg of purified RpR fragment **2** was obtained.

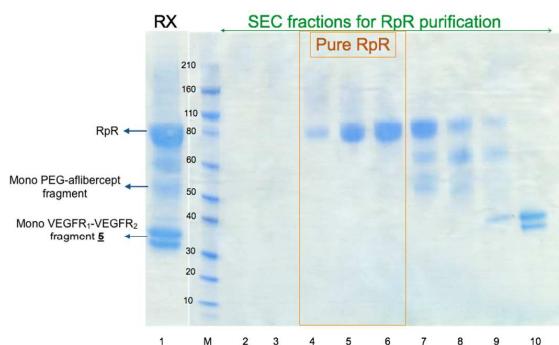


Figure 2S. SDS-PAGE gels of reaction mixture between VEGFR₁-VEGFR₂ dimer **6** (1.0 mg/mL) and PEG reagent **3** (1 eq, 10 kDa), purification of RpR using size-exclusion chromatography (SEC); Novex Bis-Tris 4-12% gel stained with colloidal blue for protein. *M*: standard protein markers, *Lane 1*: Conjugation reaction mixture, *Lanes 2-10*: SEC fractions for RpR purification.

Thiol-capped VEGFR₁-VEGFR₂ fragment **7**

Iodoacetimide (18 mM, 5.5 mg) was added to a solution of VEGFR₁-VEGFR₂ 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

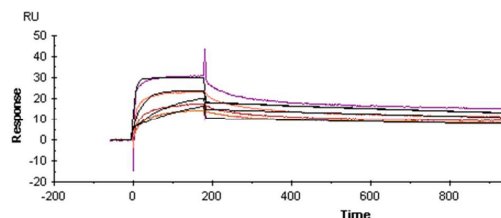
The DynoPro plate reader II (Wyatt technology) measures the size (hydrodynamic radius) and size distribution of the protein from a 0.5 nm to 1000 nm range using a dynamic light scattering (DLS) method. Samples (aflibercept, VEGFR₁-VEGFR₂ dimer **6**, thiol capped VEGFR₁-VEGFR₂ fragment **7** and RpR **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 °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, VEGFR₁-VEGFR₂ dimer **6** and RpR **2**. Human recombinant VEGF₁₆₅ (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 °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.

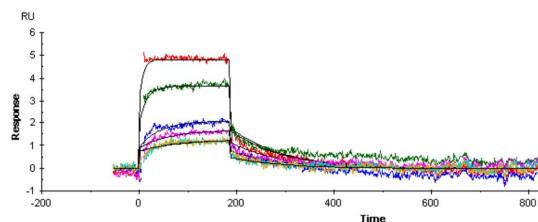
Equilibrium dissociation constants (affinity) were calculated from the rate constants ($K_D = k_d / k_a$). Figure S3 show representative fitting curves for aflibercept and RpR using 1:1 binding model.

(A) Aflibercept



tc	Chi ² (RU ²)	U-value
3.34×10^{15}	0.823	5

(B) RpR 2



tc	Chi ² (RU ²)	U-value
1.59×10^{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 VEGFR₁-VEGFR₂ dimer 6 and VEGFR₁-VEGFR₂ monomer 7 illustrates the increased binding in aflibercept due to bivalency.

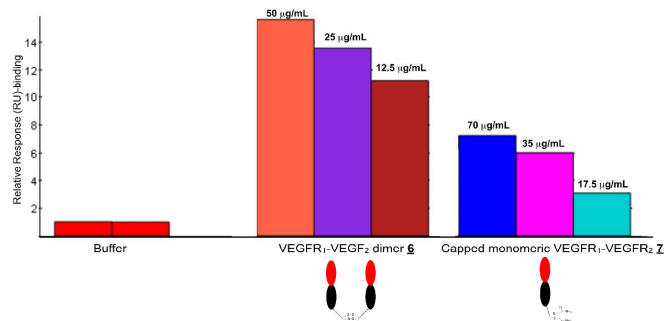


Figure S4. Binding chart for VEGFR₁-VEGFR₂ dimer 6 and thiol-capped monomeric VEGFR₁-VEGFR₂ 7.

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 VEGF₁₆₅ 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 $\mu\text{g/mL}$ of RpR 2, and 0.08, 0.04 and 0.015 $\mu\text{g/mL}$ of aflibercept were required. The samples were pre-incubated with hVEGF₁₆₅ for 2 h at 37 °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-TNF α IgG (0.08 $\mu\text{g/mL}$). Triplicate wells were prepared for each test environment. The assay plate was then placed in a humidified incubator (37 °C, 5% CO₂). 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 °C), followed by alkaline phosphatase-linked goat anti-mouse secondary antibody (1:500 dilution, 0.5 mL per well, 60 min at 37 °C). Cells were then rinsed and permanently stained for CD31 using insoluble 5-bromo-4-chloro-3-indolyphosphate/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.

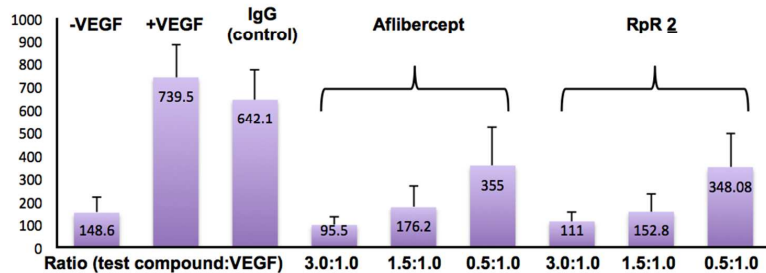
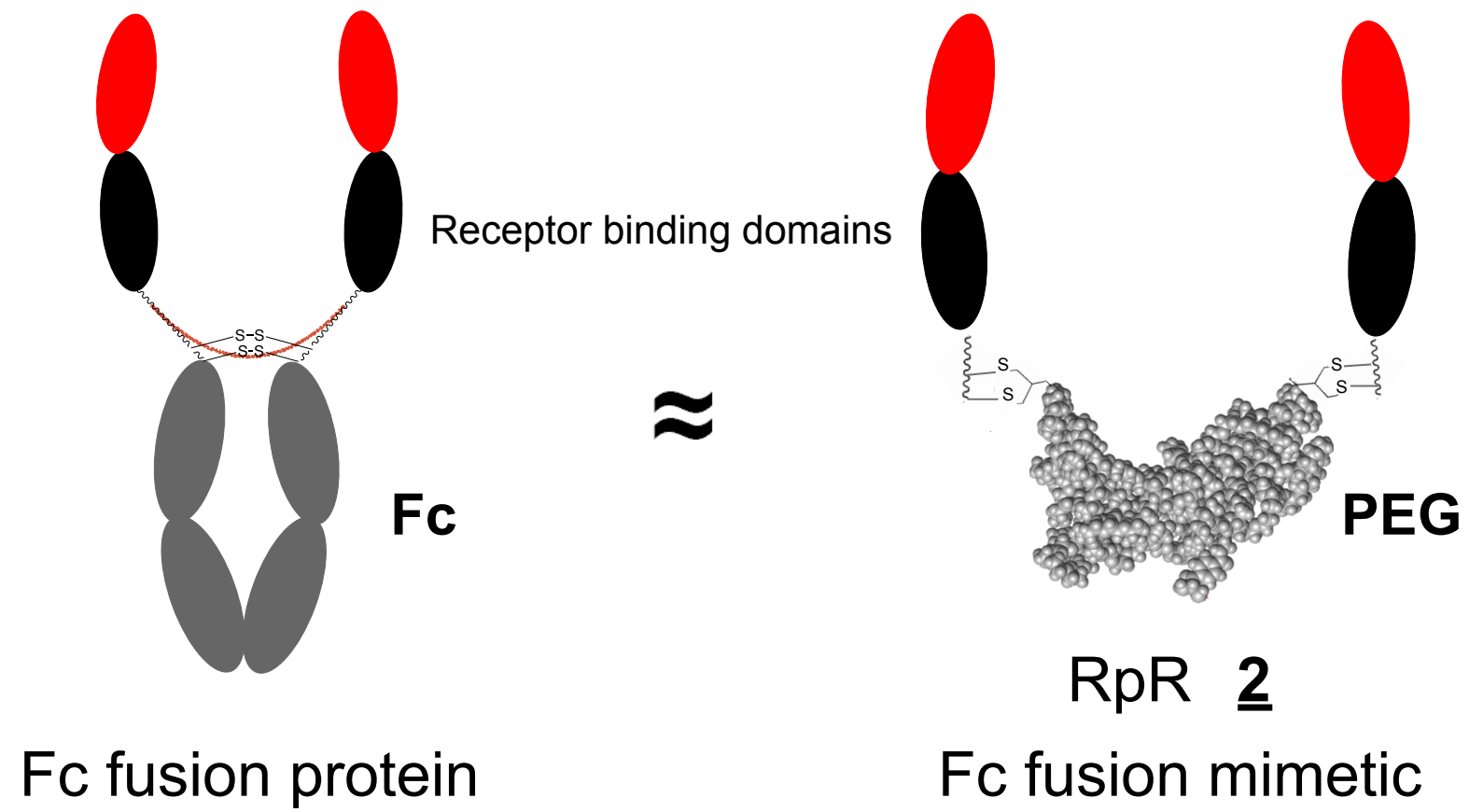


Figure S5. Tubule formation observed for medium alone, medium + VEGF, Anti-mouse TNF- α IgG + VEGF, aflibercept + VEGF and RpR + VEGF. Ratios are the amount of test compound to VEGF. VEGF was present at a fixed concentration of 10 ng/mL.

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Journal Name

COMMUNICATION

Fc-fusion mimetics

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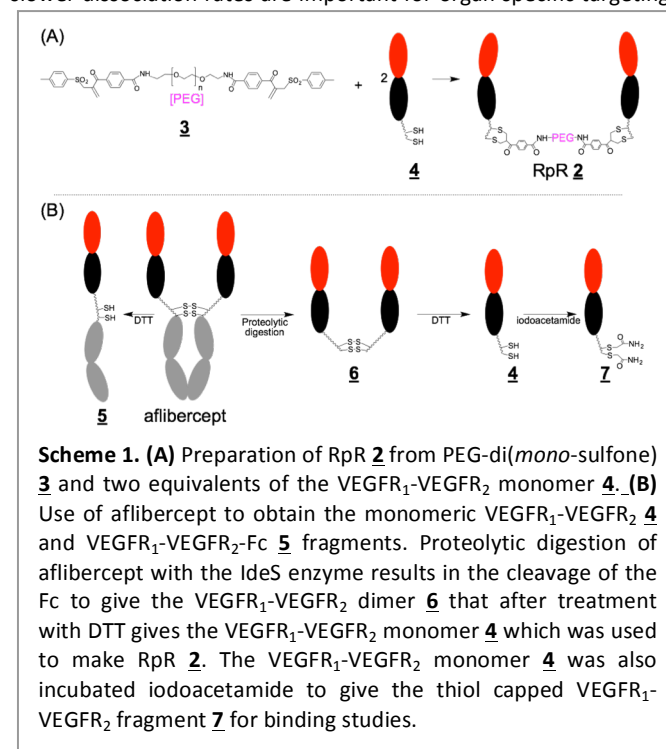
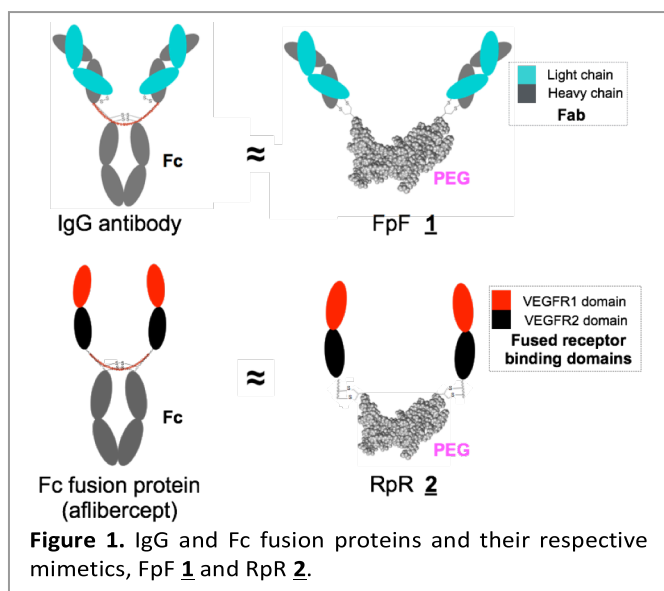
DOI: 10.1039/x0xx00000x

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The Fc-fusion mimetic RpR **2** was prepared by disulfide bridging conjugation using PEG in the place of the Fc. RpR **2** displayed higher affinity for VEGF than aflibercept. This is caused primarily by a slower dissociation rate, which can prolong a drug at its site of action. RpRs have considerable potential for development as stable, organ specific therapeutics.

To achieve effective bivalency and high affinity, the two Fabs in an IgG antibody are mobile and are linked together as if each Fab (or protein) is bound at the end of linear molecule (Figure 1). FpFs **1** are IgG antibody mimetics (Figure 1) designed to have enhanced stability and binding properties compared to IgGs. They are prepared from PEG-di(mono-sulfone) **3** and two antibody fragments (Fabs).¹ Fc-based fusion proteins² (Figure 1) are also capable of

exploiting the therapeutic advantages of bivalency that are displayed by IgGs. Several Fc-fusion proteins are registered for clinical use³ and they will continue to offer considerable clinical potential because of Fc recycling, but they can be difficult to produce during early preclinical research and to scale for production.⁴ Fc-fusion proteins are also often prone to aggregation during downstream processing⁵ and have similar stability limitations as IgGs. There are therapeutic applications where the Fc is not needed or can cause problems.⁶ One area of interest is the use of antibody based medicines in organ specific applications such as the eye. In such cases, Fc recycling does not occur and effector function can be deleterious, especially in the treatment of inflammatory conditions. Improved stability is important to formulate more concentrated solutions to decrease the frequency of dose administration and improved binding properties such as slower dissociation rates are important for organ specific targeting.



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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

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In an effort to further explore the potential of antibody-based mimetics that are made using the PEG-di(*mono*-sulfone) **3** (Scheme 1A, Figure 1) we describe an Fc-fusion mimetic that we call RpR **2**, for receptor binding region-PEG-receptor binding region.

Aflibercept (Eylea) is a Fc-fusion protein that is used to treat age related macular degeneration (AMD) by binding to vascular endothelial growth factor (VEGF) in the back of the eye. It is administered by intravitreal injection directly into the eye. Fc-fusion proteins are related to IgG antibodies in that both have an Fc domain. IgG antibodies have two heavy and two light chains. The Fc-domain comprises the C2 and C3 regions of the two heavy chains. Disulfide bonds exist in IgG antibodies that are between the heavy chains in the hinge region to separate the Fc domain from the hinge and Fabs. Aflibercept is a homodimer that comprises 2 monomers of (VEGFR₁-VEGFR₂)-(C2-C3), referred to here as VEGFR₁-VEGFR₂-Fc **5** (Scheme 1B), with disulfide bonds expected to be in the peptide sequence between the target binding domains (VEGFR₁-VEGFR₂) and the Fc domain (C2-C3 regions) (Figure 1).⁷ Aflibercept is glycosylated with a total molecular weight of 115 kDa, of which 97 kDa is due to the protein component. Each mono-sulfone moiety in reagent **3** undergoes site-specific conjugation with the two cysteine thiols from a disulfide bond by a sequence of addition-elimination reactions to insert a stable 3-carbon methylene bridge between the two thiols of the original disulfide bond (Scheme S1, ESI).⁸ The thiol ether bonds in a rebridged disulfide bond are more stable than the original disulfide bond. To make the desired RpR **2** we first had to obtain the VEGFR₁-VEGFR₂ fragment **4** by proteolytic digestion of aflibercept to remove the Fc domain (Scheme 1B).

It was first confirmed that aflibercept migrated to an approximate molecular weight of about 115 kDa by SDS PAGE (Figure 2, lane 1). Aflibercept was then treated with dithiothreitol (DTT) to reduce the accessible disulfide bonds thought to exist in an hinge like region between the binding domain (VEGFR₁-VEGFR₂) and the Fc domain. A broad band appeared at ~55-60 kDa by SDS-PAGE (Figure 2, lane 2). We believe this band corresponds to the monomeric VEGFR₁-VEGFR₂-Fc **5** (Scheme 1B). Glycosylation is usually somewhat heterogeneous in therapeutic proteins, so we inferred that the broadness of the band at ~55-60 kDa was due to glycosylation heterogeneity. DTT was then removed using a PD-10 column and the reduced aflibercept solution was incubated with Ellman's reagent which indicated the presence of 4 accessible cysteine thiols in aflibercept (Figure 1S, Table 1S, ESI). This suggested that there are 2 cysteines in each VEGFR₁-VEGFR₂-Fc **5** monomer which can form two disulfide bonds in aflibercept analogous to what is found in the hinge region of IgG antibodies. Hence it was thought possible that an RpR **2** derived from aflibercept could be prepared using the PEG-di(*mono*-sulfone) **3**. If only one cysteine had been present in the VEGFR₁-VEGFR₂-Fc **5** monomer, there are stable, mono-thiol conjugation linkers available⁹ that would have been utilised in a bifunctional reagent analogous to PEG-di(*mono*-sulfone) **3**.

Proteolytic digestion of aflibercept was then examined in an effort to obtain the monomeric VEGFR₁-VEGFR₂ fragment **4** (Figure 3). Preliminary digestion studies of aflibercept using immobilised papain yielded only difficult to characterise small peptide fragments. We had previously used papain to digest IgGs to obtain

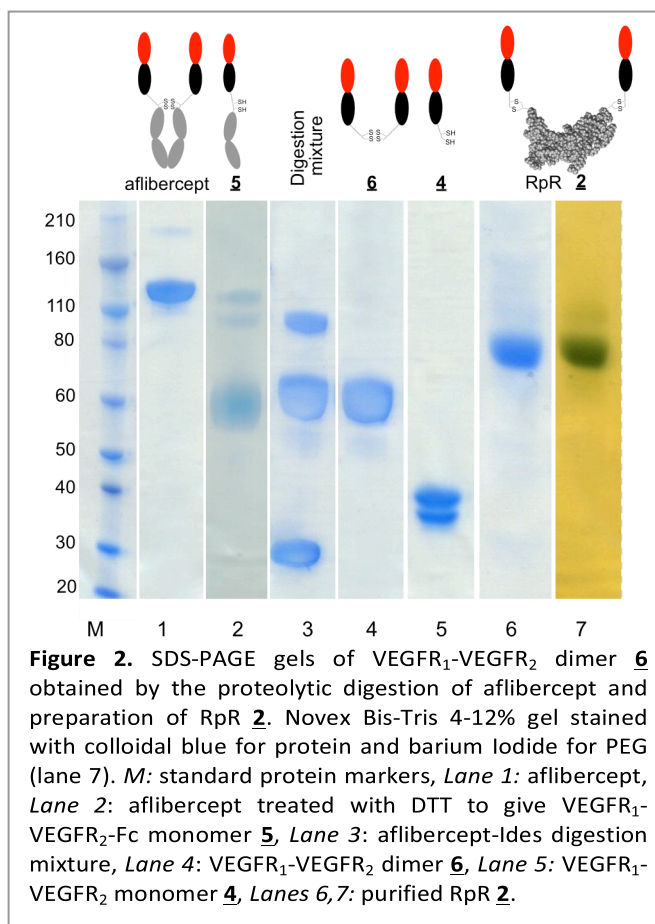


Figure 2. SDS-PAGE gels of VEGFR₁-VEGFR₂ dimer **6** obtained by the proteolytic digestion of aflibercept and preparation of RpR **2**. Novex Bis-Tris 4-12% gel stained with colloidal blue for protein and barium iodide for PEG (lane 7). *M*: standard protein markers, *Lane 1*: aflibercept, *Lane 2*: aflibercept treated with DTT to give VEGFR₁-VEGFR₂-Fc monomer **5**, *Lane 3*: aflibercept-IdeS digestion mixture, *Lane 4*: VEGFR₁-VEGFR₂ dimer **6**, *Lane 5*: VEGFR₁-VEGFR₂ monomer **4**, *Lanes 6, 7*: purified RpR **2**.

Fabs,^{1, 10} but recognised that proteolytic digestion of different antibody subclasses and motifs can be difficult to control.¹¹

A second proteolytic enzyme (IdeS enzyme, FabRICATOR[®], Genovis) that can cleave an IgG at glycine-glycine bonds¹² in the hinge reagent to give F(ab)₂ was then examined. Incubation of aflibercept with IdeS resulted in 3 bands by SDS-PAGE (Figure 2, lane 3). New bands appeared at approximately 30 kDa and 60-70 kDa. A third band at approximately 95-100 kDa in this gel was thought to be undigested aflibercept. The lower molecular weight fragment at 30 kDa, which is thought to be the cleaved Fc, and the band for undigested aflibercept were removed by eluting the digestion mixture over a column that binds to the Fc domain (CaptureSelect Midispin, Genovis). This provided a purified a non-Fc containing fragment at 60-70 kDa (Figure 2, lane 4) which was thought to be the VEGFR₁-VEGFR₂ dimer **6** (Scheme 1B).

Incubation of VEGFR₁-VEGFR₂ dimer **6** with DTT caused this fragment to disappear to give 2 lower molecular weight fragments (Figure 2, lane 5). These fragments are thought to be the desired VEGFR₁-VEGFR₂ monomer **4** (Scheme 1). Two bands are often observed after reduction of Fabs that are obtained by proteolytic digestion. This can be due to miscleavage reactions during proteolysis and may be exacerbated for aflibercept due to its difference in structure compared to an IgG and due to aflibercept glycosylation. There are five N-glycosylation sites on each monomeric VEGFR₁-VEGFR₂-Fc fragment **5** which may be partially or completely glycosylated. There may also be additional heterogeneity caused by differences in saccharide structure.

To prepare the RpR **2**, the VEGFR₁-VEGFR₂ dimer **6** was first incubated with DTT for 30 minutes to give the VEGFR₁-VEGFR₂ monomer **4** (Scheme 1B). The reaction mixture was carefully eluted over a PD-10 column to remove the DTT while avoiding disulfide reformation, and then the PEG di(mono-sulfone) reagent **3** (derived from a 10 kDa PEG precursor) was added to the solution of the monomeric VEGFR₁-VEGFR₂ **4**. Incubation of the reaction mixture for 3 h (Figure 2S, lane 1, ESI) was then followed by purification by size exclusion chromatography (Figure 2S, lanes 2-10, ESI) to give the purified RpR **2** which appeared in a band at approximately 70 kDa (Figure 2, lanes 6 and 7). Two detection dyes were used, first coomassie blue to detect protein (lane 6) and then barium iodide to detect the PEG (lane 7) being conjugated to the protein. Starting from 0.8 mg (in 1.0 mL) of VEGFR₁-VEGFR₂ dimer **6**, approximately 0.16 mg (in 0.5 mL) of RpR **2** was obtained (~ 20 % yield).

At 25°C the purified RpR **2** displayed a solution size of 10.7 ± 0.5 nm (Pd, 0.7 ± 0.1 nm), which is similar to the starting aflibercept (10.2 ± 0.7 nm; Pd, 0.6 ± 0.1 nm). The FpF antibody mimetics **1** were also a similar solution size to the corresponding IgG.¹ This is in stark contrast to when PEG is conjugated only at one terminus to a single protein where the solution size of a PEG-protein conjugate is dominated by the random coil nature of PEG.¹³ When only one terminus of PEG is conjugated to a protein, the other PEG terminus has considerable freedom to allow the PEG to maintain a large solution structure.

As a macromolecule, the RpR has a protein at each terminus of the PEG scaffold, which is analogous to an A-B-A block copolymer where it is known that the two functionalised end blocks can self-associate.¹⁴ The dimeric VEGFR₁-VEGFR₂ fragment **6** also displayed a solution size of 10.03 ± 0.1 nm (Pd, 0.7 ± 0.1 nm) which is similar to both aflibercept and RpR **2**. Interestingly, when the VEGFR₁-VEGFR₂ fragment **6** was treated with DTT and the cysteine thiols were blocked with iodoacetamide, the cysteine thiol-capped monomeric VEGFR₁-VEGFR₂ fragment **7** (Scheme 1B) displayed a solution size of 7.2 ± 0.4 nm (Pd, 0.7 ± 0.1 nm). Although the dimer **6** is twice the molecular weight of the VEGFR₁-VEGFR₂ monomer **7**, its size in solution is only about 40% larger suggesting that there may be some non-covalent intramolecular association between each of the VEGFR₁-VEGFR₂ monomers in the dimer **6**.

The binding properties of the RpR **2** and aflibercept were then evaluated by surface plasmon resonance (Biacore) to determine the affinity (K_D), and the rate constants of association (k_a) and dissociation (k_d) (Table 1). Vascular endothelial growth factor-165 (VEGF₁₆₅), which is a ligand for aflibercept, was immobilised at a density to minimise or prevent rebinding events (91 RU).^{1, 15} The dissociation rate (k_d) for the RpR **2** was slower than what was observed with aflibercept. Interestingly, the k_a appeared to be slightly faster in RpR **2** compared to aflibercept. This is in contrast to what was previously observed for anti-VEGF FpF which had a slower association rate than the precursor IgG antibody.¹ However it was the decreased k_d of RpR **2** that appeared to be the

dominating factor to cause the improved affinity of RpR **2** compared to aflibercept (Table 1). Representative fitting curves for aflibercept and RpR **2** are shown in the ESI (Figure S3, ESI).

Exploiting reduced dissociation rates may be a viable strategy to increase efficacy by increasing the residence time and mode of action within specific tissue.¹⁶ Although the reduction in k_d for FpF **1** is also slower than the parent IgG,¹ there appears to be a greater relative reduction in k_d for the RpR **2** compared to its parent Fc-fusion (i.e. aflibercept). During initial dissociation steps from the ligand of one of the two VEGFR₁-VEGFR₂ domains in the RpR **2**, PEG conformational flexibility may be more efficient for rebinding than the polypeptide linking the Fc domain to the VEGFR₁-VEGFR₂ domain in aflibercept. This suggests there is less flexibility in the bivalent binding moieties in the Fc-fusion protein (aflibercept) than there is in an IgG (e.g. bevacizumab).

The VEGF binding of the capped VEGFR₁-VEGFR₂ monomer **7** (Figure S4, ESI) was reduced when compared to the VEGFR₁-VEGFR₂ dimer **6**. This exemplified the advantages of the cooperative bivalent binding that is possible with (i) aflibercept, (ii) the dimeric VEGFR₁-VEGFR₂ fragment **6** and (iii) RpR **2** (Table 1). The similar binding properties that were observed for both the dimer **6** and aflibercept suggests that the placement of the accessible disulfide bonds linking each monomer in aflibercept is important for the mobility of the VEGFR₁-VEGFR₂ binding domains. Inclusion of a polypeptide sequence to extend the VEGFR₁-VEGFR₂ receptor domains away from the aflibercept disulfide bonds to better optimise dissociation rates would be expected to make aflibercept less stable. Such an added polypeptide sequence to increase the flexibility of the VEGFR₁-VEGFR₂ receptor domains would invariably lack secondary structure in a similar way to the hinge region of IgG antibodies. While the hinge region in IgG antibodies provides the flexibility needed for cooperative and bivalent binding of both Fabs,¹⁷ the IgG hinge region is also vulnerable to degradation and disulfide scrambling.¹⁸ The stable conjugation imparted by PEG-di(mono-sulfone) **3** and use of a PEG scaffold provides enough flexibility of the VEGFR₁-VEGFR₂ binding moieties to potentially maximise both association and dissociation rates that could be important in the development of new therapeutics.

Sample	k_a ($\times 10^5$) $M^{-1}s^{-1}$	k_d ($\times 10^{-4}$) s^{-1}	K_D (k_d/k_a) nM
Aflibercept	0.88	4.20	4.78
(VEGFR ₁ -VEGFR ₂) ₂ 6	1.20	5.20	4.30
RpR 2	1.13	1.90	1.71

Table 1. Binding kinetic constant rate of aflibercept, VEGFR₁-VEGFR₂ dimer **6** and RpR **2**, Affinity, dissociation and association rate constants were studied using CM3 chip immobilised with 91 RU human VEGF at 25°C. The range of concentrations used for the conjugates was 0.06 μM to 1.6 μM. Data were collected from 2 independent experiments for RpR **2** and 3 independent experiments for aflibercept and VEGFR₁-VEGFR₂ dimer **6**.

RpR **2** was then evaluated *in vitro* using a human umbilical vein endothelial cell (HUVEC) co-culture (Figure 3). This assay measures the migration and the formation of an anastomosing network that is characterised by tubule and junction formation during HUVEC proliferation. These processes are characteristic for angiogenesis and are often a good *in vitro* measurement for angiogenesis.¹⁹ RpR **2** and aflibercept were incubated with VEGF₁₆₅ at different molar ratios of 3.0, 1.5, 0.5 for 2 hours at 37°C prior to incubation with HUVECs. VEGF₁₆₅ and anti-mouse TNF-α IgG were used for positive controls. Images were obtained after fixing HUVECs with an anti-CD31 antibody to differentiate between the endothelial tubular network and non-endothelial structures of similar apparent morphology (Figure 3A). These images suggest that both aflibercept and RpR **2** have similar anti-angiogenic properties. Quantification of tubule (Figure S5, ESI) and junction formation (Figure 3B) (AngioSys Image Analysis Software, TCS Cellworks Ltd.) showed that the formation of these structures were similarly inhibited in a concentration dependent manner by both RpR **2** and aflibercept.

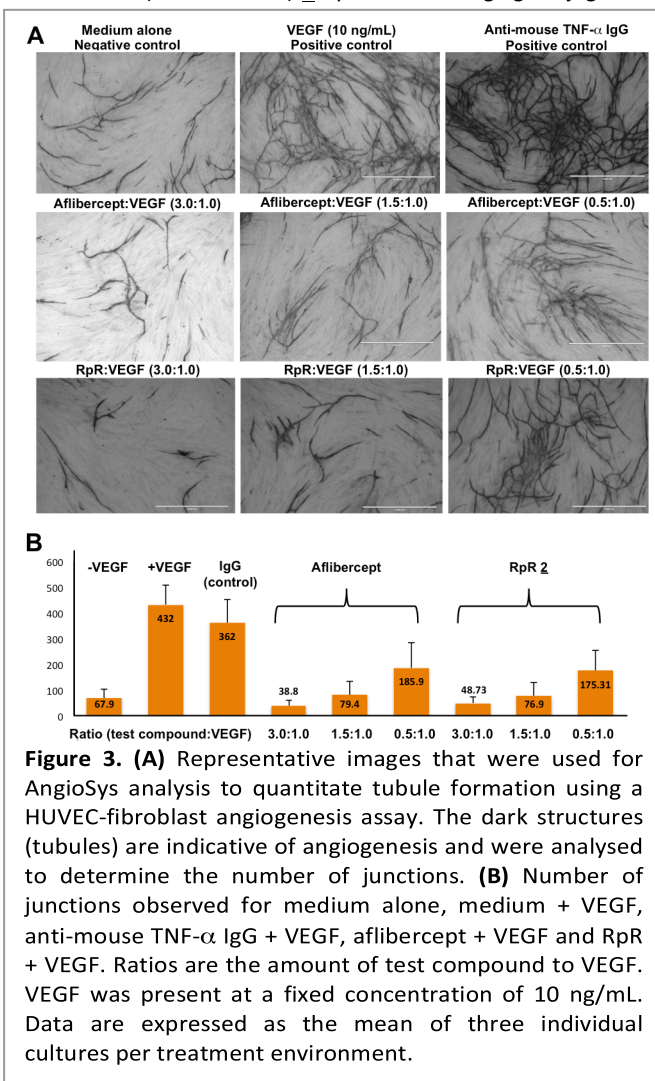
To summarise, a new antibody Fc-fusion mimetic called an RpR was prepared. Aflibercept is a clinically used Fc-fusion protein that targets VEGF was used for these studies. Proteolytic digestion of aflibercept followed by incubation with DTT provided the monomeric VEGFR₁-VEGFR₂ domain **4** that was then conjugated to the PEG-di(mono-sulfone) **3** by disulfide bridging conjugation to

give the anti-VEGF RpR **2**. The strategy to proteolytically digest aflibercept provided the means to compare the properties of the RpR Fc-fusion mimetic **2** with aflibercept. The solution size of RpR **2** and its *in vitro* activity are comparable to aflibercept. Of most interest is that binding studies show that RpR **2** has higher affinity for VEGF compared to aflibercept primarily due to a slower dissociation rate. Antibody based mimetics such as RpR **2** have potential for development as stable, organ specific therapeutics.

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