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Storage Stability and Solution Binding Affinity of an Fc-Fusion Mimetic

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Abstract

This study evaluates the storage stability and solution binding affinity of a novel Fc-fusion mimetic, receptor-PEG-receptor (RpR), designed to address limitations of the current therapeutic aflibercept, a gold-standard therapy for age-macular degeneration (AMD). Using di(*bis*-sulfone) PEG linker as a structural scaffold, the mimetic aims to improve the storage stability and binding efficacy of the Fc fusion protein. Mass photometry and size-exclusion chromatography demonstrated that RpR, even in an unformulated buffer, exhibits superior storage stability exceeding 10 months compared to aflibercept. Furthermore, microscale thermophoresis was employed to determine RpR's binding affinity to VEGF in solution, providing a more physiologically relevant assessment than traditional binding assays. These findings highlight RpR's potential as a therapeutic candidate for the treatment of AMD disease, warranting further investigation.

Introduction

The development of therapeutic proteins, particularly Fc-fusion proteins, has significantly advanced the treatment of many chronic diseases, including autoimmune disorders, cancers, and ocular diseases [1-3]. Fc-fusion proteins combine the functional domain of a biologically active protein with the Fc region of an antibody [3]. They offer therapeutic advantages through bivalency similar to IgGs, but they can be difficult to produce during early preclinical research and to scale for production [4]. They are also prone to aggregation during downstream processing and have similar stability concerns as IgGs [5, 6]. In certain therapeutic contexts, such as organ-specific applications like ocular treatments, the Fc region may be unnecessary or even detrimental, particularly in managing inflammatory conditions [7]. This highlights the need for alternative formats that retain the benefits of Fc-fusion proteins while addressing their limitations.

Aflibercept (Eylea) is a prominent example of an Fc-fusion protein that has revolutionised the treatment of neovascular age-related macular degeneration (AMD), diabetic macular edema, and other retinal vascular diseases. Aflibercept functions by binding to vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), preventing these factors from interacting with their receptors on the surface of endothelial cells and thereby inhibiting pathological angiogenesis and vascular permeability [8]. Despite its success, aflibercept faces several challenges, including stability issues and the need for frequent intravitreal injections, which can lead to patient discomfort and increased risk of complications [9, 10].

Structurally, aflibercept shares similarities with IgG antibodies due to the presence of an Fc domain. However, unlike IgG antibodies with their two heavy and two light chains, aflibercept is a homodimer glycoprotein consisting of two identical monomers. Each monomer is composed of vascular endothelial growth factor receptor 1 ($VEGFR_1$) and vascular endothelial growth factor receptor 2 ($VEGFR_2$) binding domains fused to the C2 and C3 regions of the Fc domain ($VEGFR_1$-$VEGFR_2$-Fc) [8]. These binding domains are linked by a disulfide bond, as shown in Figure 1. The protein molecular weight of aflibercept is 96.9 kDa and contains 15% glycosylation sites to give a total molecular weight of 115 kDa. It is a highly glycosylated protein, where 4 sites are located in the VEGFR domain and 1 site is located in the Fc domain [11].

To modify this structure, a di(*bis*-sulfone) PEG reagent can specifically target and react with the disulfide bond's cysteine thiols. This reaction replaces the disulfide bond with a stable 3-carbon methylene bridge. The PEG di(*bis*-sulfone) linker allows for site-specific conjugation, providing structural stability while preserving the functional integrity of the protein.

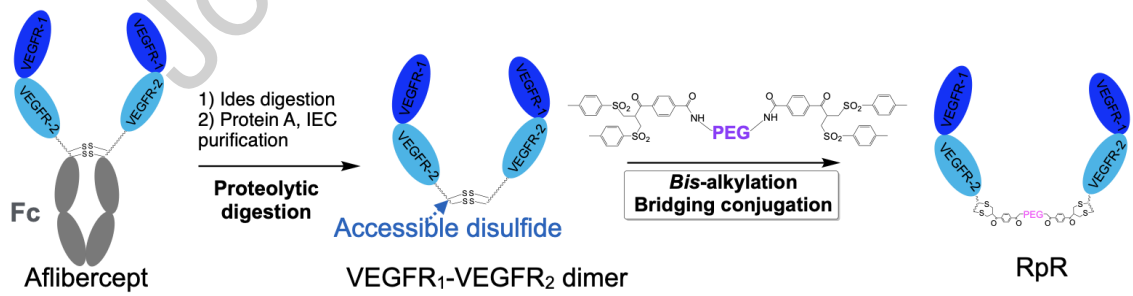


Figure 1. Preparation of RpR from aflibercept using bis-alkylation briding conjugation. The workflow shows the stages of RpR preparation beginning with the isolation of the $VEGFR_1$-$VEGFR_2$ dimer using proteolytic digestion and bis-alkylation bridging conjugation using PEG di(*bis*-sulfone) linker to produce RpR.

Previously, we developed a novel Fc-fusion mimetic, termed Receptore-PEG-Receptor (RpR) [12], and antibody mimetic termed Fab-PEG-Fab (FpF) [13], utilising a PEG di(*bis*-sulfone) linker as a scaffold to achieve bivalency and high affinity. FpFs and RpR are designed to replace PEG with the Fc where two binding domains are linked together as if each binding domain is bound at the end of a linear molecule. FpF as IgG antibody mimetic and RpR as Fc-fusion mimetics are designed to have enhanced stability and binding properties compared to their parents' antibodies (Figures 1, 2). Indeed, FpFs demonstrated superior protein stability compared to the parent IgG, with no aggregation or light and heavy chain dissociation observed in either liquid or lyophilised forms [14]. However, the long term storage stability and binding properties of RpR in solution have not yet been investigated, which is the primary focus of this study.

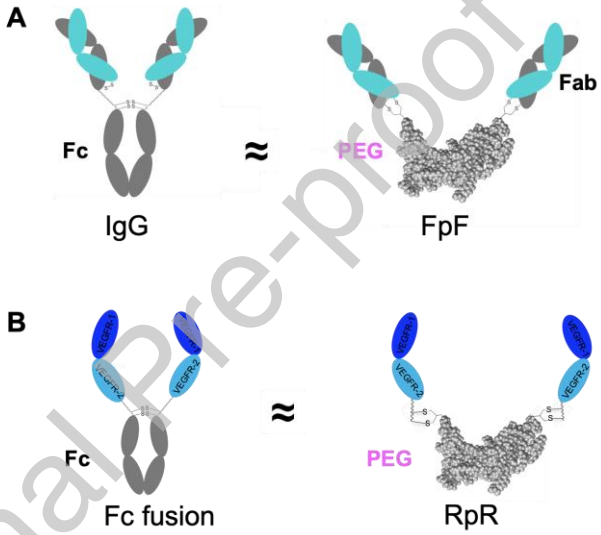


Figure 2. Structure of **(A)** IgG versus FpF, **(B)** Fc-fusion (aflibercept) versus RpR.

In our earlier studies, we investigated the binding affinity of RpR using surface plasmon resonance (SPR) technology, where RpR's interaction with immobilised VEGF was analysed. These initial findings demonstrated promising binding characteristics and lower binding affinity for RpR compared with aflibercept [12]. However, SPR immobilises one binding partner, which does not fully replicate the dynamic interactions that occur in a physiological environment where both molecules are free in solution.

The primary objective of this current study is two-fold: firstly, to assess the stability of RpR under various storage conditions, including storage (4°C) and physiological (37°C) temperatures, and secondly, to evaluate its binding affinity to VEGF when both molecules are in solution, closely mimicking the conditions within the human body. Stability is a critical factor for the efficacy and safety of therapeutic proteins, as instability can lead to aggregation, denaturation, and loss of function. These changes can reduce therapeutic

effectiveness and increase the risk of adverse immune reactions. To address these objectives, we employed mass photometry to assess the stability of RpR over time at different temperature, tracking any potential aggregation or degradation. Mass photometry offered by Refeyn Ltd. is a relatively new technology that provides highly specific information about the mass and stoichiometry of biomolecules in solution. It is important to note that while mass photometry provides valuable insights into species distribution, it may over-represent smaller fragments due to their faster diffusion to the sensor. However, in this study, mass photometry served as a valuable tool for rapidly assessing the overall stability profiles of RpR and aflibercept at 37 °C. The primary goal is to compare their relative stability under different storage conditions, and mass photometry complemented by SDS-PAGE and size-exclusion chromatography (SEC) allowed us to track changes in the abundance of different species over time. Additionally, microscale thermophoresis (MST) was utilised to measure the binding affinity of RpR to VEGF in solution, providing a more physiologically relevant assessment compared to the previous SPR analysis.

The results indicate that RpR exhibits superior storage stability and solution binding affinity, positioning it as a promising candidate for further development in ocular inflammation therapies. These findings support the continued exploration of PEG di(*bis*-sulfone) conjugation as a valuable tool in the design of next-generation therapeutic proteins. In summary, By addressing the limitations of current treatments, such as aflibercept, we hope to pave the way for more effective and patient-friendly therapies for ocular and other chronic diseases.

Results and Discussion

RpR preparation: Building upon previous work, aflibercept was subjected to proteolytic digestion using IdeS, cleaving below the hinge region while preserving the essential disulfide bridge for site-specific conjugation. This yielded a $VEGFR_1$-$VEGFR_2$ dimer (Figure 3, lane 3), further purified using size exclusion chromatography (SEC) to prepare it for subsequent conjugation.

The site-specific conjugation process involved reducing the $VEGFR_1$-$VEGFR_2$ dimer to monomers using DTT as a reducing agent. Following removal of excess DTT via a PD-10 desalting column, the monomers were incubated with the PEG di(*bis*-sulfone) reagent (Figure 3, lane 4). The resulting RpR was purified using SEC (Figure 3, lane 5) and treated with sodium triacetoxyborohydride (STAB). This incubation aids in reducing the reactive ketone group to a more stable hydroxyl group [15], thereby minimising potential side reactions and improving the overall stability of the RpR conjugate.

SDS-PAGE analysis confirmed that aflibercept migrated to an approximate molecular weight of 115 kDa (Figure 3, lane 2). Incubation of aflibercept with IdeS enzyme (FabRICATOR, Genovis) specifically cleaved the Fc-fusion protein at the glycine-glycine bonds within the hinge region, yielding a $VEGFR_1$-$VEGFR_2$ dimer with a molecular weight of approximately 60 kDa. This was confirmed by SDS-PAGE analysis (Figure 3, lane 3). Incubation of the $VEGFR_1$-$VEGFR_2$ dimer with DTT resulted in the reduction of the disulfide bond, yielding two lower molecular weight fragments of approximately 30 kDa each. These fragments are thought to be the desired $VEGFR_1$-$VEGFR_2$ monomer, each possessing a free thiol group available for subsequent bis-alkylation conjugation. The RpR conjugate was prepared by adding PEG di(*bis*-sulfone) reagent (1 equivalent) to the $VEGFR_1$-$VEGFR_2$ monomer and 3 hours incubation at ambient temperature. Subsequent purification using SEC yielded the purified RpR, which migrated as a single band at approximately 70-80 kDa on SDS-PAGE (Figure 3, lane 5).

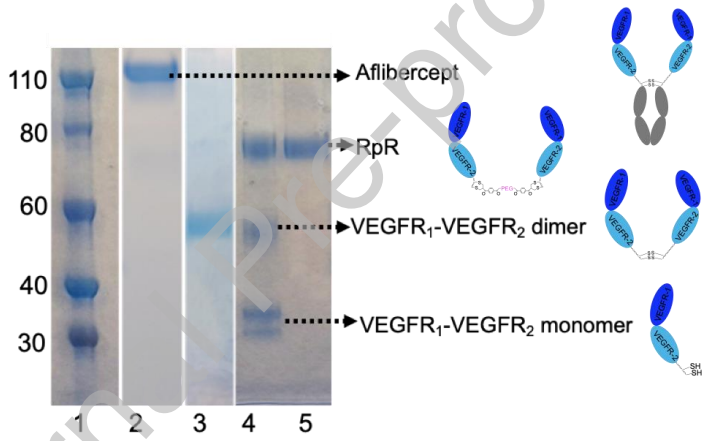
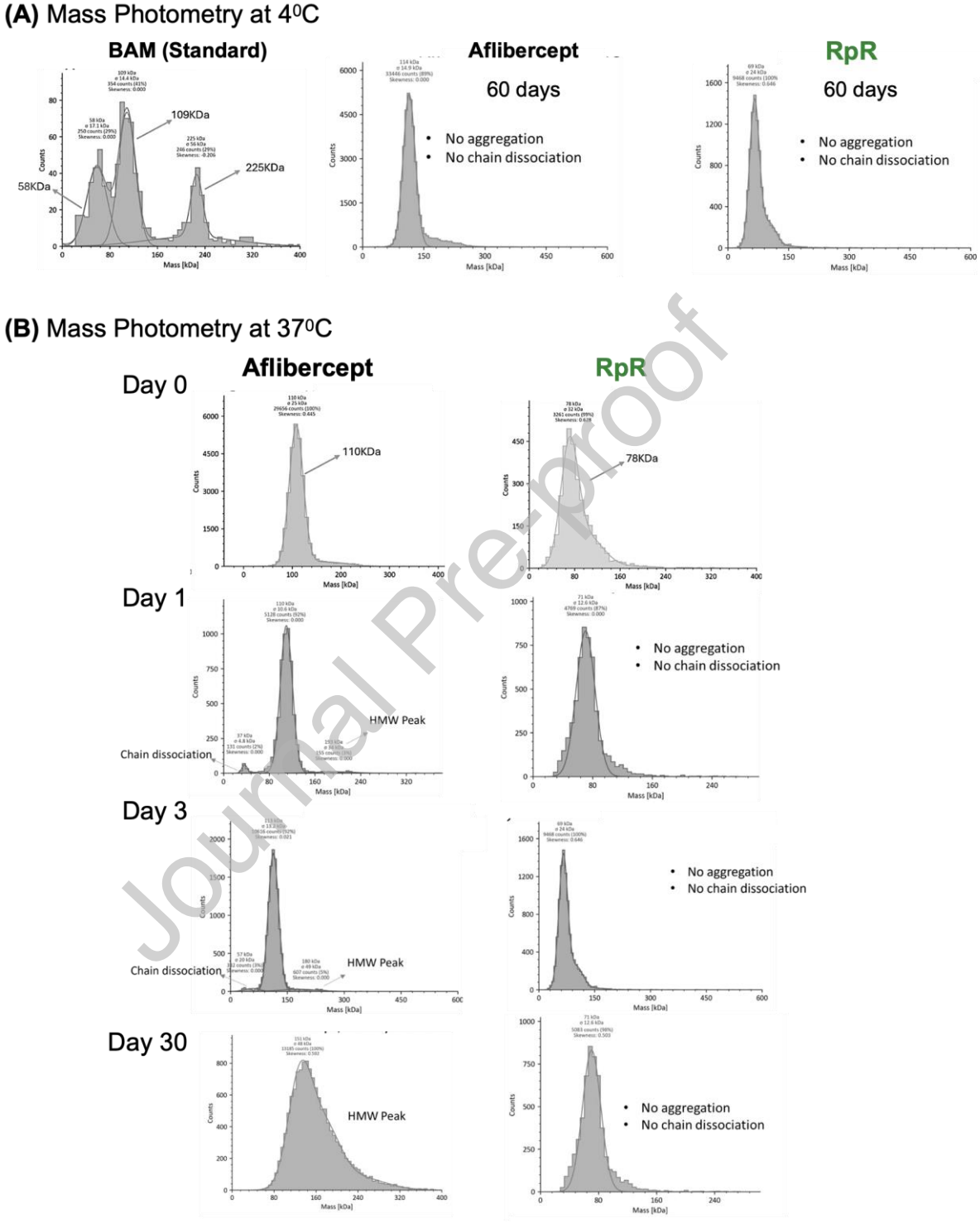


Figure 3. Representative SDS-PAGE analysis for RpR preparation using di(*bis*-sulfone) PEG reagent. SDS-PAGE was stained with instant blue for protein staining. ***lane 1***: Novex pre-stained protein marker, ***lane 2***: Aflibercept (115 kDa), ***lane 3***: $VEGFR_1$-$VEGFR_2$ dimer resulted from Ides digestion of aflibercept, ***lane 4***: Reaction mixture between PEG di(*bis*-sulfone) reagent (1 eq) and reduced-$VEGFR_1$-$VEGFR_2$ dimer after 3 hours incubation at ambient temperature, The slight difference in molecular weight between the two $VEGFR_1$-$VEGFR_2$ monomers might be attributed to variations in glycosylation [11]. ***lane 5***: Purified RpR.

RpR Stability Study: To assess the stability of RpR compared to aflibercept, both molecules were subjected to accelerated degradation studies by incubation at 37°C for 1, 3, and 30 days. Mass photometry, a single-molecule imaging technique, was employed to monitor the structural integrity and potential aggregation of the proteins over time [16, 17]. This method

allows for the direct visualisation and quantification of individual protein molecules based on their mass, providing valuable insights into their stability under physiological conditions.



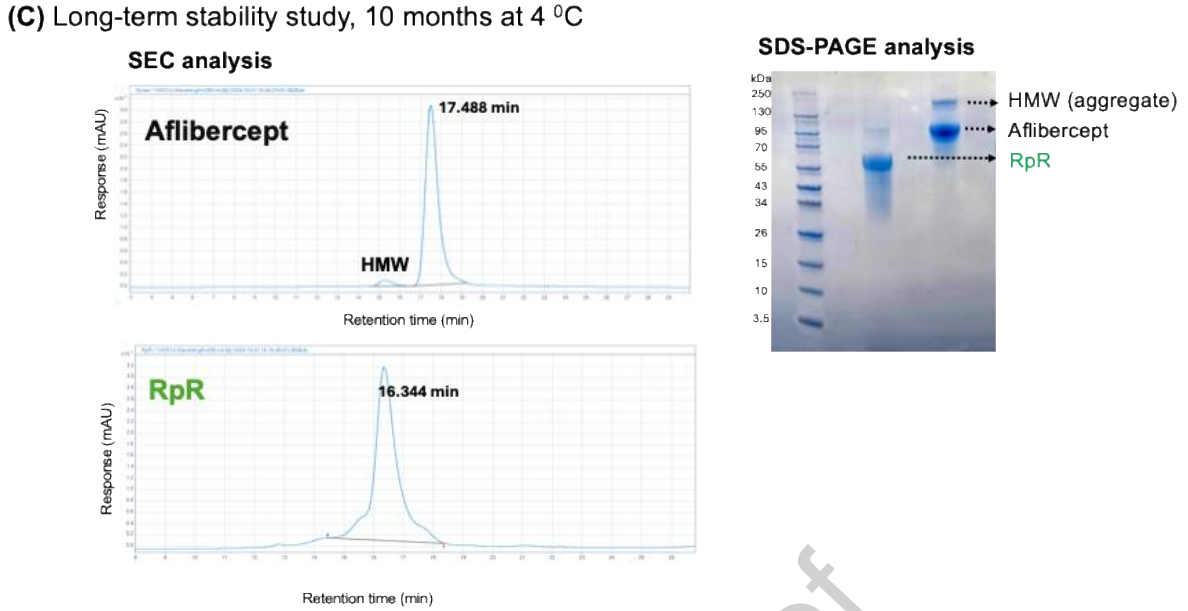


Figure 4. (A and B) Mass photometry analysis of aflibercept (115 kDa) and RpR (78 kDa) stability. BAM (ß-amylase) is used as a standard which exists in solution as a mixture of monomers (58 kDa), dimers (109 kDa), and tetramers (225 kDa). **(A)** at 4^0C after 60 days, both aflibercept and RpR remain stable, showing no aggregation or chain dissociation. **(B)** at 37^0C after 30 days, RpR maintained its stability, while aflibercept displays high molecular weight (HMW) species and chain dissociation after 24 hours. **(C)** Size-exclusion chromatograohy (SEC) and SDS-PAGE analysis to determine the stability of aflibercept (0.24 mg/mL in formulated buffer) and RpR (0.24 mg/mL in PBS only) after storage at 4 ^{0}C for 10 months. SEC analysis of aflibercept revealed a prominent high molecular weight (HMW) peak, indicative of aggregation, while RpR appeared as a single peak. SDS-PAGE analysis confirmed the presence of HMW species in the aflibercept sample, further demonstrating the superior stability of RpR under these storage conditions.

Mass photometry analysis (Figure 4, A) revealed a single peak for RpR at 78 kDa, confirming the purity and solution stability of the prepared conjugate. This result is consistent with the SDS-PAGE analysis (Figure 3, lane 5), and previously published MALDI-ToF anlysis which also showed a single band at approximately 70-80 kDa, further validating the successful purification and preparation of RpR. BAM (ß-amylase) serves as a standard in mass photometry, offering multiple, distinct mass peaks that facilitate the calibration of the mass photometer's response.

Both RpR in an unformulated buffer (PBS only) and aflibercept (in its formulated buffer) exhibited stability at a concentration of 40mg/mL when stored at 4°C for a minimum of 60 days (Figure 4, A). This finding aligns with previous reports on repackaged ziv-aflibercept, demonstrating maintained stability at 4°C for up to 60 days [9]. Upon incubation at 37°C, aflibercept exhibited early signs of instability, showing trace amounts of aggregation (high molecular weight peak) and chain dissociation even after 24 hours (Figure 4B). Conversely, RpR demonstrated remarkable stability, maintaining a single peak at 78 kDa for

the entire 30-day incubation period at 37°C. Long-term stability was studied using size-exclusion chromatography (SEC) and SDS-PAGE analysis. Both RpR (in PBS only) and aflibercept (formulation solution) were stored at 4 ^{0}C for 10 months and then analysed (Figure 4, C). SEC and SDS-PAGE analysis revealed the presence of a high molecular weight peak (HMW) for aflibercept, indicative of aggregation. In contrast, RpR appeared as a single peak, demonstrating superior stability under these storage conditions.

Interestingly, RpR eluted earlier than aflibercept in SEC despite its lower molecular weight. It is important to note that SEC separation can be influenced by the specific column used, including the properties of the stationary phase. Variations in stationary pore size distribution and column material could lead to differences in elution profiles for the same molecules. AdvanceBio SEC column (2.7 µm) was used to analyse the aggregation and/or degradation in RpR compared with aflibercept. This column has a unique hydrophilic coating to minimise secondary interactions between the sample and stationary phase [18]. It is plausible that RpR exhibits minimal interaction with the column material, leading to faster elution, while aflibercept may experience some weak interactions that slightly delay its progress.

This enhanced stability of RpR is likely attributed to the replacement of the hinge region with the PEG di(*bis*-sulfone) linker. This flexible, 3-carbon bridge formed between cysteine residues, along with the formation of thio-ether bonds, imparts greater resilience to the molecule, preventing aggregation and chain dissociation. Similar stability improvement has been observed in FpF molecules compared to their parent IgG counterparts [14], suggesting that this modification strategy may be a broadly applicable approach for improving the stability of protein therapeutics.

RpR Binding Affinity in solution: Microscale thermophoresis (MST) was employed to evaluate the binding affinity of RpR and aflibercept to $VEGF_{165}$ in solution, offering a more physiologically relevant assessment compared to SPR techniques. MST's advantage lies in its ability to measure interactions in a free solution environment, eliminating potential artefacts caused by immobilisation and providing a more accurate representation of binding behaviour *in vivo* [19, 20].

In this study, $VEGF_{165}$ was fluorescently labelled using an amine coupling reaction. While amine coupling is a widely used labelling method for MST, it's important to acknowledge its potential limitations. Heterogeneous labelling and steric hindrance due to the attached dye could influence the measured binding affinity [21]. However, in this study, we chose to label VEGF to maintain consistency and minimise variability between the aflibercept and RpR binding measurements. VEGF. This approach also facilitated a direct

head-to-head comparison between of the binding affinities of RpR and aflibercept to VEGF. Labelling either RpR or aflibercept would have introduced potential bias due to the non-specific nature of amine coupling in the labelling process. This could potentially interfere with the binding sites, leading to inaccurate affinity measurements.

The degree of labeling (DOL) of $VEGF_{165}$ was optimised at 0.56 to ensure a strong and specific signal while minimising potential interference with the binding interaction. A range of concentrations of both RpR and aflibercept (0.9 µM to 0.00011 µM) was tested against a constant concentration of labelled $VEGF_{165}$ (0.625 µM) in MST capillaries to generate binding curves and accurately determine the binding affinity (KD) for each interaction. Figure 5 demonstrates the binding curves and corresponding binding affinity for the interaction of both RpR and aflibercept with $VEGF_{165}$.

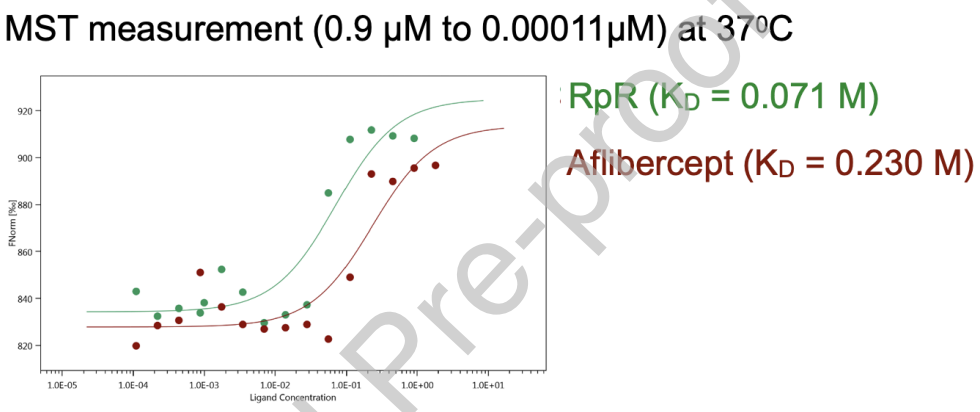


Figure 5. Microscale thermophoresis analysis comparing the binding affinities of aflibercept (red dots) and RpR (green dots) to $VEGF_{165}$. Serial dilutions of aflibercept and RpR (0.9 µM to 0.00011 µM) were tested against a constant concentration of fluorescently labelled $VEGF_{165}$ (0.625 µM). The resulting dose-response curves illustrate the binding behaviour and affinity (KD) of each molecule. Data points represent the mean of three independent experiments.

MST dose-response curves revealed that RpR had a lower dissociation constant (K_D = 0.071 M) compared to aflibercept (K_D = 0.230 M), indicating a higher binding affinity for $VEGF_{165}$. This observation is consistent with the SPR data [12], which also demonstrated a slower dissociation rate (kd) for RpR compared to aflibercept, and higher binding affinity (K_D) indicating a longer residence time on the target. The enhanced affinity of RpR might be attributed to the replacement of the hinge region with a flexible PEG linker. This modification may provide greater conformational freedom for the $VEGFR_1$-$VEGFR_2$ domains to interact optimally with $VEGF_{165}$, thus facilitating stronger and more stable binding. The flexibility of the PEG linker might also contribute to a faster association rate (ka) observed in the SPR data [12, 13], allowing RpR to bind more rapidly to $VEGF_{165}$ compared to aflibercept.

The combination of MST and SPR data provides a comprehensive understanding of the binding kinetics and affinity of RpR and aflibercept towards $VEGF_{165}$. The results highlight the potential of modifying the linker region to enhance the binding properties of antibody-based drugs, offering a promising strategy for improving their therapeutic efficacy. The increased affinity of RpR, combined with its favourable stability profile, suggests that it may be a more potent and effective therapeutic option compared to aflibercept for the treatment of diseases mediated by $VEGF_{165}$. Previously published *in-vitro* angiogenesis assays of human umbilical vein endothelial cell (HUVEC) co-cultures indicated that RpR and aflibercept behave similarly in anti-angiogenic bioassays [12]. However, a more comprehensive set of *in vitro* and *in vivo* studies will be required to fully characterise RpR's anti-angiogenic and pharmacological properties.

Conclusion

Building upon our previous successful generation and characterisation of RpR, this study further demonstrates its enhanced stability and binding affinity to VEGF. By replacing the Fc domain with a flexible PEG linker, we achieved several key advantages. First, we observed improved storage stability compared to the parent aflibercept, as evidenced by mass photometry analysis after incubation at 37 °C, and SEC and SDS-PAGE analysis of RpR stored in PBS buffer for over 10 months at 4 °C. This analysis revealed the remarkable long-term stability of RpR with no evidence of aggregation or PEG dissociation. Second, this modification reduces the potential for immunogenicity and avoids Fc-mediated effector functions. While these functions can be desirable in some therapeutic contexts (e.g., cancer immunotherapy), they are undesirable in the treatment of ocular diseases, where the eye is an immune-privileged site. Activating these effector functions in the eye could lead to inflammation and tissue damage. The substitution of the Fc domain with PEG in RpR aligns with the therapeutic goal of neutralising VEGF without triggering unwanted immune responses. This is particularly important considering the chronic nature of AMD and the need for repeated intravitreal injections.

In summary, the replacement of the Fc domain with a PEG scaffold in RpR offers a promising strategy for treating ocular diseases by mitigating immune-related side effects, potentially improving long-term safety and efficacy, and enhancing storage stability. This approach holds significant promise for addressing the limitations of current protein-based drugs and expanding the therapeutic landscape for a wide range of diseases.

Experimental section

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

Aflibercept (6.0 mg) was donated from Moorfield Eye Hospital after patient injections. It was digested using immobilised IdeS enzyme (FabRICATOR®, FragIT MidiSpin, Genovis; Cat no A0-FR6-100), following the optimised protocol reported previously in [12] and the manufacturer's instruction. Breifly, the column was equilibrated with cleavage buffer (50 mM sodium phosphate, 150 mM NaCl, pH 6.6) and aflibercept (6 mg in 1.0 mL cleavage buffer) was loaded onto the column. The digestion was incubated for 30 min at room temperature with end-over-end mixing. The resulting digestion mixture was then purified using a CaptureSelect MidiSpin column (Genovis) packed with a multi-species Fc affinity matrix, according to the manufacturer's instructions. The purified $VEGFR_1$-$VEGFR_2$ dimer was eluted, analysed by SDS-PAGE, and quantified using a micro BCA assay, yielding 2 mg of purified protein.

Preparation of RpR general procedure

Dimeric $VEGFR_1$-$VEGFR_2$ fragment (0.8 mg) was reduced with dithiothreitol (DTT, 6.0 mM) in PBS (pH 7.3) for 30 minutes at room temperature to yield monomeric $VEGFR_1$-$VEGFR_2$. The reaction mixture was buffer exchanged into sodium phosphate buffer (20 mM), EDTA (10 mM), pH 7.6 using a PD-10 column to remove excess DTT. The monomeric $VEGFR_1$-$VEGFR_2$ (0.24 mg/mL, 3.3 mL) was then conjugated with 0.9 equivalents of a 10 kDa PEG di(*bis*-sulfone) reagent (previously described [12, 13]) for 12 hours at 4°C. The resulting RpR was purified by size exclusion chromatography (Superose 12 HR 10/30 column, PBS mobile phase) and fractions containing the desired product were pooled, concentrated, and quantified by micro BCA assay, yielding 0.2 mg of purified RpR.

Determination of the mass size of RpR by mass photometry

The mass photometry (Refeyn Ltd) was used to monitor molecular weight, aggregation, and degradation of the protein over time. Samples (aflibercept and RpR) were prepared at a concentration of 0.3 mg/mL using PBS buffer, which was passed through a 0.2 µm filter. Briefly, samples and standards (β-Amylase and BSA, both 100 nM) were diluted in PBS and analyzed using a mass photometer. The instrument was prepared by applying immersion oil to the objective lens and positioning magnets on the stage. Samples were introduced using the droplet dilution method (2 µL sample in 18 µL PBS) and analysed for 60 seconds, during which molecular weight and particle counts were recorded. Stability samples were analysed at specified time intervals following incubation.

Determination of stability using Size-Exclusion Chromatography and SDS-PAGE analysis

Size-exclusion chromatography (SEC) was conducted to evaluate the stability of RpR and aflibercept, specifically to detect any degradation or chain dissociation occurring over 10

months of storage at 4°C. The samples included RpR at a concentration of 0.24 mg/mL and aflibercept, diluted to a final concentration of 0.24 mg/mL in PBS. Samples were injected into an Agilent 1260 Infinity HPLC system, using PBS (pH 7.3) as a mobile phase and a flow rate of 0.35 mL/min. Each run was completed over 35 minutes, and separation was achieved using an AdvanceBio SEC column (300Å, 2.7 µm, 7.8 x 300 mm). Detection was monitored at 280 nm to accurately capture any potential structural changes or dissociations in the protein samples.

Non-reducing SDS-PAGE was employed to analyse protein samples without disrupting disulfide bonds. Samples (20µL) were mixed with a non-reducing sample buffer (4µL, Pierce™ LDS Sample Buffer, Non-Reducing (4X)), and loaded (10µL) loaded into precast SDS-PAGE gels (Novex™ Tris-Glycine Mini Protein Gels, 4–20%, 1.0 mm). Electrophoresis was conducted at a constant voltage of 160 V for 1 hour using NuPAGE™ MOPS SDS Running Buffer (20X). The gels were stained with Coomassie blue for protein staining and a silver staining kit (Pierce™ Silver Stain Kit) to visualise any trace quantity of protein.

Determination of the solution binding affinity of RpR by microscale thermophoresis

Microscale thermophoresis (Monolith, NanoTemper) was used to determine the binding affinity of aflibercept, and RpR to human recombinant $VEGF_{165}$ (38 kDa). $VEGF_{165}$ (10 µM) was labelled with a fluorescent dye according to the manufacturer's instructions (Protein Labeling Kit RED-NHS 2nd Generation (Amine Reactive), Nanotemper) and purified using the provided purification column (Monolith premium capillaries, included in the kit). Briefly, $VEGF_{165}$ (10 µM in labelling buffer) was incubated with the RED-NHS dye (300 µM in labelling buffer containing 50% DMSO) for 30 minutes at room temperature in the dark. The labelled protein was purified using the provided gravity flow column (Monolith premium capillaries, NanoTemper Technologies) with phosphate-buffered saline (PBS) as the elution buffer. The degree of labelling (DOL), defined as the ratio of dye molecules to protein molecules, was determined spectrophotometrically by measuring the absorbance at 205 nm (protein) and 650 nm (dye) and was optimised to fall within the range of 0.5-1.0 for optimal MST signal. A systematic approach was used to optimise the DOL by varying the dye-to-protein ratio in a series of small-scale labelling reactions and assessing the DOL after each purification.

For the MST assay, serially diluted RpR and aflibercept samples were prepared in PBS (0.9µM to 0.00011µM) and mixed with labelled $VEGF_{165}$ (0.15 µM) in glass capillaries. MST measurements were performed using a Monolith Pico system with MST power of 40% and excitation power of 80%. The dissociation binding constant (KD) for each interaction was determined using the MST analysis software.

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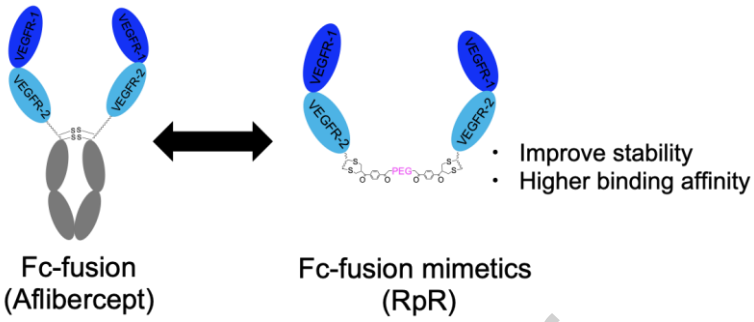
Graphical Abstract



Declaration of Competing Interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: