Supplementary Materials

Methods

Turbidity and lysis assays

Human plasma system

To study the effect of Affimer proteins on fibrin clot formation and lysis, human normal plasma was diluted 1 in 6 and incubated with Affimer protein in 100 mM NaCl, 50 mM Tris, pH 7.4, at different concentrations (molar ratios of Affimer:fibrinogen of 1:1, 5:1, and 10:1). Samples were incubated for 30 minutes at ambient temperature before being added to a 96-well plate in duplicate. After 30 min incubation, a lysis mix containing 83 ng/mL tissue plasminogen activator (tPA; TC Technoclone, Surrey, UK) or 20 nM urokinase type plasminogen activator (uPA; TC Technoclone, Surrey, UK) (all final concentrations) was added to allow lysis, and an activation mix containing 7.5 mM $CaCl_2$ and 0.03 U/mL thrombin (Merck, Hertfordshire, UK) was added to start the reaction. Measurements of the optical density at 340 nm were taken at 12 second intervals on a Multiskan Go plate reader (Thermo Scientific, Loughborough, UK) over a period of 2 hours. Maximum absorbance, a measure of fibrin clot density and/or fiber thickness was determined for each sample. Lysis time was calculated as the time taken from maximum absorbance to the time at which the maximum absorbance was reduced by 50% as described previously (1).

Plasma samples were initially tested using commercially available human normal mixed pool plasma (First Link UK Ltd., Birmingham, England), followed by testing individual samples from healthy controls to investigate inter-individual variability.

Moreover, we tested samples deficient in FVIII, representing high risk bleeding groups (Haematologic Technologies Inc., Essex, UK). A mixture of tissue factor and phospholipids at a final concentration of 0.5-10 pM (PPP-Reagent HIGH, Thrombinoscope, Maastricht, The Netherlands) was used to initiate thrombin generation. Clotting time in these experiments was calculated as the time taken to reach clot maximum absorbance.

Purified system

We applied the same principles used in plasma samples but with some practical modifications. Purified fibrinogen (Calbiochem, Feltham, UK) was used at 0.5 mg/mL (1.47 µM) (final concentration) instead of plasma. The activation mix used in a purified system contained 0.05 U/mL thrombin and 2.5 mM $CaCl_2$ whereas the lysis mix contained 39 ng/mL tPA and 3 µg/mL plasminogen (Enzyme Research Laboratories, Swansea, UK). Purified turbidimetric assays were performed with Affimer protein at 1.47 µM, 7.35 µM and 14.7 µM, equivalent to 1:1, 5:1 and 10:1 Affimer to fibrinogen molar ratio, respectively. Experiments comparing the effect of plasminogen/tPA with plasmin lysis of clots were performed by forming clots with fibrinogen and Affimer protein, adding thrombin and calcium, and allowing clotting to proceed for one hour. After one hour, a lysis mix was overlaid onto the formed clots. Lysis mix contained either tPA (39 ng/mL) and plasminogen (3 µg/mL) or plasmin (3 µg/mL).

Laser scanning confocal microscopy (LSCM)

Plasma was diluted 1 in 4 in 100 mM NaCl, 50 mM Tris, pH 7.4, and incubated with Affimer proteins (Affimer:fibrinogen molar ratios of 1:1, 5:1 and 10:1), with the addition of Alexa 488-labelled fibrinogen at approximately 5% (0.105 µM) (Thermo Fisher Scientific/Life

Technologies, Loughborough, UK) for 30 minutes at ambient temperature. Following incubation, an activation mix containing 0.05 U/mL human thrombin (Merck Chemicals Ltd, Nottingham, UK) and 5 mM $CaCl_2$ was added. The mixture was then loaded onto an Ibidi slide (Applied Biophysics, New York, USA). The clots were visualized on a LSM880 microscope (Carl Zeiss, Welwyn Garden City, Hertfordshire, UK) using 40 x 1.4 oil objective lens. Three Z stacks of each clot were taken, with a range of 20.3 µm at intervals of 0.7 µm (30 slices total).

Scanning electron microscopy (SEM)

Plasma clots were prepared by diluting plasma 1 in 2 in 100 mM NaCl, 50 mM Tris, pH 7.4 with the addition of Affimer protein at 1:1, 5:1 and 10:1 Affimer:fibrinogen molar ratio. Plasma clots were prepared for SEM as previously described (30). Briefly, samples were clotted with the addition of 0.5 U/mL thrombin and 2.5 mM $CaCl_2$, incubated in a humidity chamber for 2 hours, washed with sodium cacodylate buffer and fixed in 2% glutaraldehyde overnight. Stepwise dehydration in acetone and sputter coating with 5 nm iridium were then performed and the clots were visualized and photographed using a Hitachi SU8230 high performance cold field emission (CFE) SEM (Chiyoda, Tokyo, Japan) in three different areas of each clot.

Clot permeation

To study the permeation properties of fibrin clots, human plasma was incubated with Affimer protein at Affimer:fibrinogen molar ratio of 5:1 for 30 minutes at room temperature, followed by the addition of activation mix containing 1 U/mL thrombin and 10 mM $CaCl_2$. The mix was immediately transferred to 'clotting' tips (burette tips) and incubated for 2 hours at room temperature in a humidity chamber. Clotting tips were connected via plastic tubing to a reservoir of permeation buffer (100 mM NaCl, 50 mM Tris, pH 7.4) with a pressure drop of 4 cm. The flow rates of buffer were measured as previously described (2) and the Darcy constant (Ks) was calculated for each sample.

ELISA based binding assay to determine Affimer K_{DS}

Nunc-Immuno MicroWell 96 well-plates (Thermo Scientific, Loughborough, UK) were coated with 100 µL of 5 µg/mL fibrinogen (Calbiochem, Feltham, UK) in 50 mM sodium carbonate pH 9.6 and incubated at 4°C overnight. Wells were washed with Tris-buffered saline with 0.1% (v/v) Tween-20 (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) (TBST) and blocked with TBS containing 3% (w/v) bovine serum albumin (BSA) for 1 hour at 37°C. After being washed, a dilution series of Affimer protein in 1% BSA TBST was added to the wells in duplicate and incubated for 1 hour at 37°C. The wells were washed again with TBST and then incubated with mouse anti-His antibody (Roche, Welwyn Garden City, UK) diluted in 1% BSA TBST for 1 hour at 37°C and then further washed. Wells were incubated with HRP-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark), washed and reacted with water-soluble substrate for HRP (OPD tablets, Dako, Glostrup Denmark). The reaction was stopped by the addition of 1.5 M H_2SO_4 and the absorbance at 490 nm was read on a Multiskan Go plate reader (Thermo Scientific, Loughborough, UK).

ELISA based binding assay to investigate interaction of tPA, plasminogen and Affimer protein with fibrin DD fragment

Binding assays were performed as above, but plates coated with 5 µg/mL DD fragment from fibrin (Quadratech Diagnostics, UK) in TBS in place of fibrinogen. Dilution series of Affimer protein (0-5000 nM), plasminogen (0-40 µg/mL) or tPA (0-40 µg/mL) in 1% BSA TBST were added to the wells in duplicate and incubated for 1 hour at 37°C. Affimer was detected with mouse anti-His antibody (Roche, Welwyn Garden City, UK), plasminogen with goat anti-plasminogen-HRP antibody (Enzyme Research Laboratories, Swansea, UK) and tPA with mouse anti-tPA antibody (Enzyme Research Laboratories, Swansea, UK) diluted in 1% BSA TBST. For detection, Affimer and tPA wells were incubated with HRP-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark). Binding signal was detected with OPD tablets as above.

Competitive binding assays with Affimer F5/scaffold and tPA or plasminogen were performed as above, with a concentration series of Affimer protein (0-10,000 nM) incubated with either 1.25 µg/mL plasminogen or 1.25 µg/mL tPA before adding to a DD fragment-coated plate. tPA or plasminogen binding to DD in the presence of Affimer protein was detected as above.

ELISA based binding assay to investigate Affimer interaction with tPA and plasminogen

ELISAs were performed as above, except plates were coated with 5 µg/mL plasminogen or tPA. A concentration series of Affimer F5 or scaffold (0-10,000 nM) was incubated with the tPA or plasminogen, and Affimer detected as previously described.

Biacore SPR binding assay

IF-1 purified human fibrinogen (Calbiochem) (5 µg/mL in 0.1 M sodium acetate buffer, pH 5.6) was immobilized to 2000 RUs by amine-coupling using an NHS/EDC-activated CM5 chip followed by deactivation with ethanolamine/HCl using a Biacore 3000 (GE Healthcare, Buckinghamshire, UK). The running buffer for binding assays was 100 mM NaCl, 50 mM Tris, 2mM $CaCl_2$, 0.1% (v/v) Tween-20, pH 7.4. A reference surface was prepared by activating and deactivating the dextran without the addition of protein. An additional fibrin surface was prepared by injecting thrombin (1U/mL) diluted in running buffer over immobilized fibrinogen at 2 µl/min for 45 minutes to convert fibrinogen to fibrin. The fibrin surface was washed to remove thrombin cleaved fibrinopeptides A and B by injecting 1 M NaCl, 50 mM Tris, pH 7.4 at 30 µL/min.

Binding assays were performed in running buffer. Affimer proteins were diluted in running buffer to a concentration range (12.5-800 nM) for 120 seconds at 50 µl/min in three replicate experiments. The surface was regenerated by flowing running buffer for 15 minutes sufficient to return to baseline. Data were processed by subtraction of sensorgrams from the reference flow cell and a buffer injection over the derivatised surface. Affinities and rate constants for association and dissociation were analysed using a 1:1 Langmuir binding model with BIAevaluation 3.1 software.

Pull down assays for identification of F5-fibrinogen interactions

Fibrinogen (Calbiochem) (0.5 mg/mL in 50 mM Tris, 100 mM NaCl, pH 7.4) was incubated with 50 µg Affimer F5 protein prior to the addition of 35 nM plasmin (Enzyme Research Laboratories, Swansea, UK) for 1 hour at 37 °C to generate fibrinogen degradation products (FDPs). Digestions were stopped with 40 nM aprotinin (Sigma, Gillingham, UK). F5-FDPs were incubated for 30 minutes at room temperature with His-tag isolating beads (Dynabeads, Thermo Fisher, Loughborough, UK) in binding buffer (50 mM Tris, 100 mM NaCl, 0.01% (v/v) Tween-20, 20 mM imidazole, pH 7.4). Beads were washed three times with washing buffer (50 mM Tris, 500 mM NaCl, 0.01% Tween-20, 20 mM imidazole) on a magnetic rack prior to elution with binding buffer with the addition of imidazole to 300 mM. Elutions were analyzed by SDS-PAGE on 4-12% Bis-Tris gels (Thermo Fisher Scientific, Loughborough, UK). Gels were stained with GelCode Blue gel stain (Thermo Fisher Scientific, Loughborough, UK) to visualize F5-binding FDPs. Samples not containing Affimer protein F5 served as a control for non-specific binding of fibrinogen to His-tag isolating beads. Mass spectrometry (LC-MS/MS) to identify gel bands was performed by the Cambridge Centre for Proteomics (3).

Plasmin generation assay

The rate of plasmin generation by tPA was performed according to a modified protocol by Bobbink et al. (4). 96 well plates were coated with 0.5 mg/mL (1.47 µM) fibrinogen, with Affimer proteins F5 and G2 at 1.47 µM, 7.35 µM and 14.7 µM, equivalent to 1:1, 5:1 and 10:1 Affimer to fibrinogen molar ratio, respectively. Affimers were added to plates in assay buffer (40 mM Tris, 75 mM NaCl, 0.01% Tween-20 (v/v)). FXIII (3.7 µg/mL) and plasminogen (94 nM) were added, prior to the addition of an activation mix containing thrombin (0.5 U/mL) and $CaCl_2$ (2.5 mM). After 15 minutes of clotting at 37 °C, a lysis mix containing tPA (25 ng/mL) and chromogenic substrate S2251 (Chromogenix, Surrey, UK) (0.8 mM) was added. Control experiments were similarly performed, in the absence of fibrinogen with Affimer F5 at 14.7 µM. S2251 hydrolysis was monitored via kinetic absorbance readings at 405 nm every 30 seconds for five hours at 37°C. Additional experiments were performed to determine if Affimer F5 had a direct effect on tPA or plasmin activity, compared with a control protein (Affimer directed against SUMO protein). To assess tPA activity, Affimer (7.35 µM) and tPA (20 ng/mL) were incubated for 15 minutes at 37 °C, prior to the addition of chromogenic substrate S2288 (Chromogenix, Surrey, UK) (2 mM) and absorbance measured over time at 405 nm. To investigate an effect of Affimers on plasmin activity, Affimer (7.35 µM) and plasmin (90 nM) were incubated for 15 minutes at 37 °C, prior to the addition of chromogenic substrate S2251 (0.8 mM), and absorbance measured over time at 405 nm.

Figures

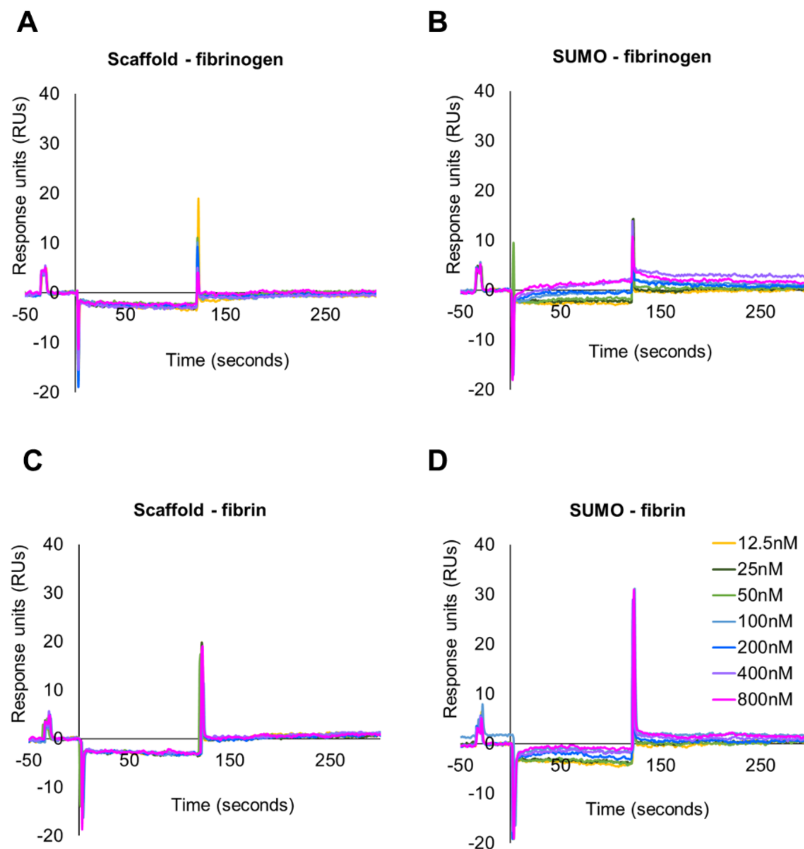

Figure S1. Affimer scaffold and a control Affimer do not bind to fibrinogen or fibrin. (A,B) Binding of Affimer scaffold and a control protein (Affimer directed against SUMO protein) to fibrinogen and (C,D) fibrin using Biacore SPR. Affimer proteins (12.5-800 nM) were injected over a fibrinogen or fibrin derivatised surface. Three independent experiments were performed, representative data are shown.

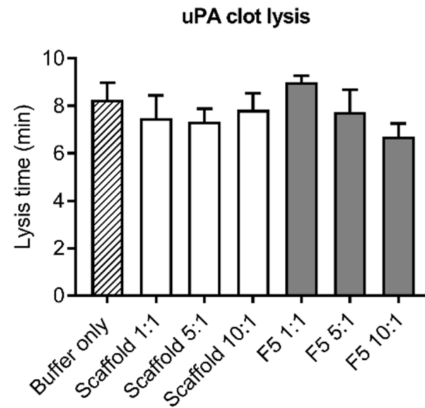

Figure S2. Effect of Affimer protein F5 or scaffold on urokinase type plasminogen activator-initiated clot lysis in human plasma. Turbidimetric experiments were performed in normal pool plasma with increasing concentrations of Affimer F5 or scaffold control protein. Clot lysis was induced with urokinase type plasminogen activator. Numbers on the x-axis represent Affimer protein:fibrinogen molar ratio. Data are presented as the mean±SD of three independent experiments. Statistical analysis was performed using one-way ANOVA comparing each molar ratio to buffer-only control.

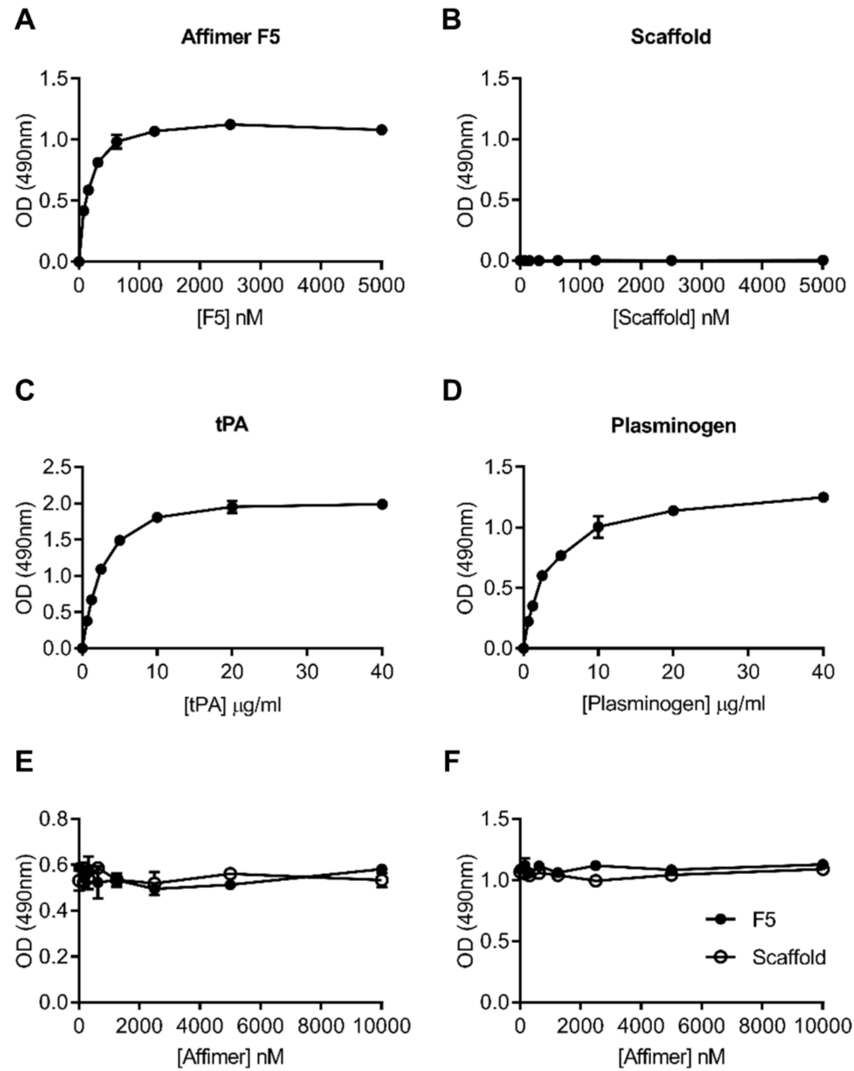

Figure S3. Binding of tPA and plasminogen to the fibrin DD fragment is not affected by Affimer F5 protein. Binding of tPA and plasminogen to fibrin DD fragment in the presence of Affimer F5 was assessed by ELISA. (A) Affimer F5 (B) Affimer scaffold (C) tPA (D) plasminogen binding to fibrin DD fragment was assessed. (E) A competition assay was performed in which wells were coated with fibrin DD fragment, prior to incubation with increasing concentration of Affimer F5 or scaffold control protein (0-10,000nM), and a constant concentration of tPA (1.25µg/mL). tPA binding to DD was detected. (F) A competition assay was performed similarly to that described in (E), with plasminogen (1.25µg/mL) in place of tPA, and plasminogen detected. All assays were performed 3 times, mean±SD of a single representative experiment is presented.

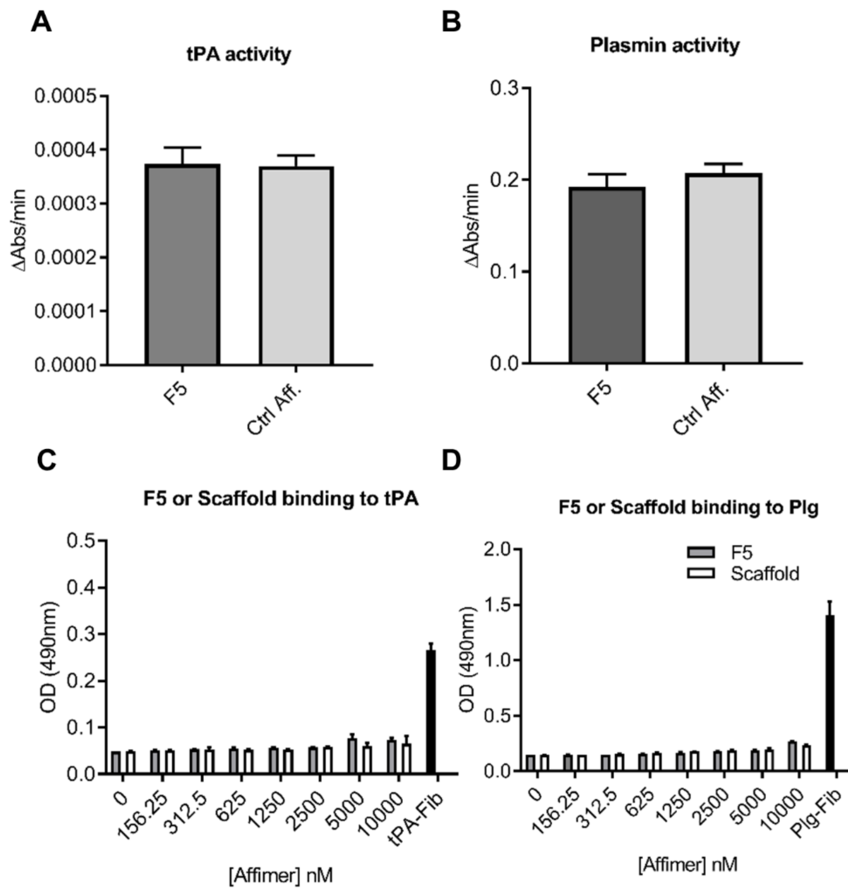

Figure S4. Affimer protein F5 had no direct effect on tPA or plasmin activity, and did not bind to tPA or plasminogen. (A) The effect of Affimer F5 on tPA activity was assessed using chromogenic substrate S2288. F5 or control (ctrl) Affimer (an Affimer directed against SUMO protein) was incubated with tPA and the hydrolysis of chromogenic substrate measured over time. (B) The effect of Affimer F5 on plasmin protein activity was also assessed, using chromogenic substrate S2251. Samples containing F5 or control Affimer were incubated with plasmin prior to the addition of chromogenic substrate. Results are presented as rate of chromogenic substrate hydrolysis (au/min). Data are presented as the mean±SD of three independent experiments. Statistical analysis was performed using two-tailed, unpaired Student *t*-tests, comparing F5 to control Affimer. (C) Binding assays were performed to investigate any direct interaction of Affimer protein with tPA or (D) plasminogen (plg). Plates were coated with (C) tPA or (D) plasminogen, and incubated with a concentration series of Affimer F5 or scaffold protein. F5 and scaffold protein were detected after incubation. Positive control in (C) is coated with tPA and received fibrinogen (Fib) in place of Affimer (black bar), in (D), plasminogen coat received fibrinogen (Fib) in place of Affimer sample (black bar). Data presented is mean±SD of a single representative experiment, which was repeated three times.

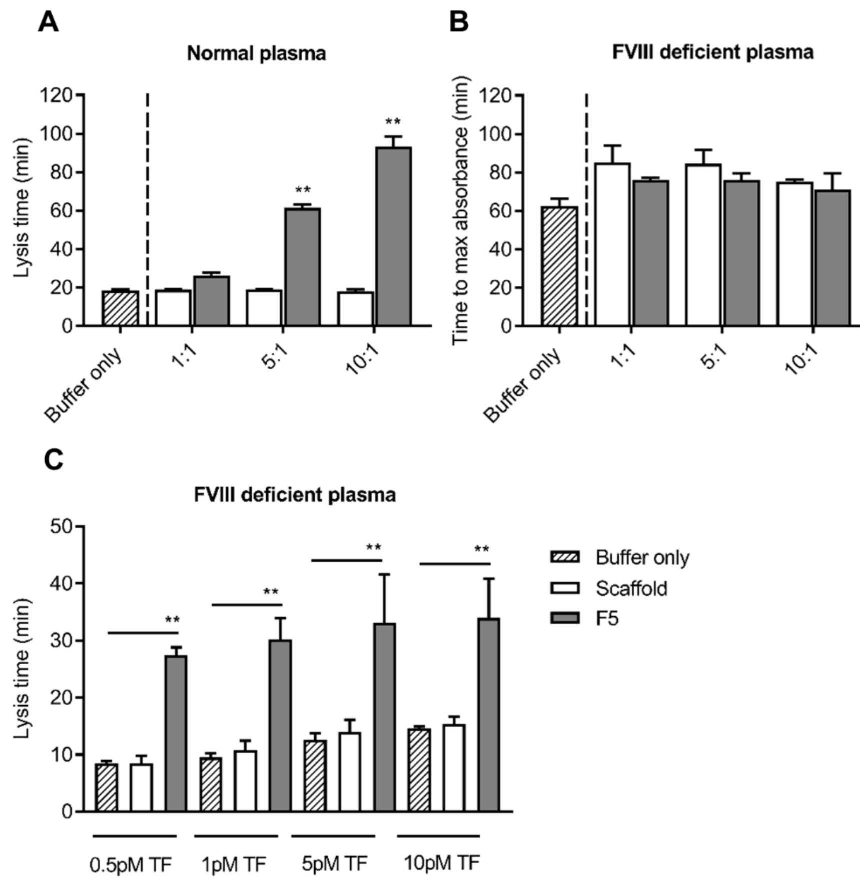

Figure S5. The effect of Affimer F5 on tissue factor initiated clotting and lysis in normal pool and FVIII deficient plasmas. (A) The effect of increasing concentrations of Affimer F5 or scaffold control protein on lysis time in normal pool plasma in which clotting was initiated with 5 pM tissue factor. (B) The effect of Affimer F5 or scaffold control on clotting time (time taken to reach maximum absorbance) in assays initiated with 5 pM tissue factor. Numbers on the x-axis represent Affimer protein:fibrinogen molar ratio. (C) A range of tissue factor concentrations (0.5-10 pM) were used to initiate thrombin generation, and lysis time calculated in the presence of Affimer protein F5 or scaffold control at 10:1 Affimer:fibrinogen molar ratio. Data are presented as the mean±SD of three independent experiments. Statistical analysis was performed using one-way ANOVA, ** represents difference from buffer-only control, $^{**}P < .01$.

References

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