Modulation of fibrin clot lysis and potential future clinical implications

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1. The introduction contains material from my review article: **Kearney K**, Tomlinson D, Smith K, Ajjan R. Hypofibrinolysis in diabetes: a therapeutic target for the reduction of cardiovascular risk. Cardiovascular Diabetology, 2017; 16(1):34.

I designed the review, undertook the literature search and wrote the manuscript, RA designed the review and critically reviewed the manuscript, KS and DC critically reviewed the manuscript.

2. Chapter 4 of the thesis includes data from: **Katherine J Kearney**, Nikoletta Pechlivani, Rhodri King, Christian Tiede, Fladia Phoenix, Ramsah Cheah, Fraser L Macrae, Katie J Simmons, Iain W Manfield, Kerrie A Smith, Benjamin EJ Spurgeon, Khalid M Naseem, Robert AS Ariëns, Michael J McPherson, Darren C Tomlinson, and Ramzi A Ajjan. Affimer proteins as a tool to modulate fibrinolysis, stabilize the blood clot and reduce bleeding complications. Blood, 2018; 133(11):p. 1233.

I was responsible for isolating Affimer F5, planning and performing all F5 experiments and data analysis/interpretation unless otherwise stated. N.P. and I.W.M performed SPR experiments and analysed SPR data. F.P. and N.P. contributed to completing turbidimetric experiments and analysis of the data. F.P. performed whole blood platelet studies using the Multiplate Analyser and contributed to completing binding assays with DD fragment. R.C. performed permeation experiments and analysis of the data. F.L.M. performed scanning electron microscopy. K.J.S. performed molecular modelling, B.E.J.S performed flow cytometry platelet studies and analysis of the data. The paper was jointly written by myself, N.P. and R.A.

3. Chapter 6 of the thesis includes data from: Macrae FL, Duval C, Papareddy P, Baker SR, Yuldasheva N, **Kearney KJ**, et al. A fibrin biofilm covers the blood clot and protects from microbial invasion. The Journal of Clinical Investigation, 2018; 128(8):p. 3356.

I was responsible for the isolation of Affimer A2. Myself and F.L.M designed Affimer confocal experiments. F.L.M performed the confocal microscopy experiments.

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Abstract

The formation of a blood clot is vital for the prevention of blood loss following vessel injury. The haemostatic system allows the closing of damaged blood vessels, retention of blood in a fluid state and removal of clots after the restoration of vascular integrity. Blood clots are formed of a network of fibrin fibres with platelets, red and white blood cells embedded within. Imbalances and alterations in coagulation factors and the multitude elements of the coagulation cascade can impact fibrin clot properties.

Fibrin clot susceptibility to lysis has implications in both bleeding and thrombotic disorders. Formation of an intravascular obstructive thrombus can result in end organ damage, and treatment is aimed at reperfusion to regain blood flow to the affected vessels followed by prophylactic therapy to prevent re-occlusion. Conversely, in trauma or surgical associated bleeding, or that resulting from some haematological disorders, limiting fibrin clot lysis to stabilise the clot is a key aim of therapy.

Considering the potential future therapeutic benefits of targeting the fibrin network, I hypothesised that fibrinogen-specific Affimer proteins represent a tool to modulate the fibrinolytic process. The aim of this work was to investigate the use of these synthetic proteins in altering fibrin clot structure/lysis, with the long term view that they may constitute new therapeutic agents for the treatment of thrombotic and/or bleeding disorders. The objectives of my work were to i) identify fibrinogen-specific Affimer proteins, ii) investigate the effects of fibrinogen-specific Affimers on fibrinolysis, and iii) characterise the mechanistic pathways for Affimer-mediated modulation of clot lysis.

My work describes the isolation of fibrinogen-binding Affimer proteins that modulate fibrin clot lysis. Particular focus was on an anti-fibrinolytic Affimer that reduced plasmin generation in a fibrin-specific manner. Elucidation of this Affimer's mechanism of action and potential therapeutic relevance of this Affimer protein was explored.

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List of Abbreviations

°C	Degrees Celsius
2-MEA	2-mercaptoethylamine
3D	3 dimensional
α2M	α2-macroglobulin
aa	Amino acids
ADP	Adenosine diphosphate
A-fib	Afibrinogenemia
Ala	Alanine
ANOVA	Analysis of variance
APC	Activated protein C
aPCC	Activated prothrombin complex
APCE	Antiplasmin cleaving enzyme
Arg	Arginine
Asn	Asparagine
AU	Absorbance units
BB	Binding buffer
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
Carb	Carbenicillin
CFE	Cold field emission
CT	Clotting time
CVD	Cardiovascular disease
Da	Daltons
ddH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Glutamic acid
E. coli	*Escherichia coli*
EACA	Epsilon amino caproic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMBOSS	European molecular biology open software suite
F	Phenylalanine

FDP	Fibrin (or fibrinogen) degradation products
FEIBA	Factor eight inhibitor bypassing activity
FIX	Factor nine
FIXa	Activated factor nine
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
FV	Factor five
FVa	Activated Factor five
FVII	Factor seven
FVIIa	Activated factor seven
FVIII	Factor eight
FVIIIa	Activated factor eight
FX	Factor ten
FXa	Activated factor ten
FXI	Factor eleven
FXIa	Activated factor eleven
FXII	Factor twelve
FXIIa	Activated factor twelve
FXIII	Factor thirteen
FXIIIa	Activated factor thirteen
g	G force/relative centrifugal force
GFD	Growth factor domain
Glu	Glutamic acid
Gly	Glycine
GPIb-V-IX	Glycoprotein Ib-V-IX
GPIIb/IIIa	Glycoprotein IIb/IIIa
GPVI	Glycoprotein VI
H	Histidine
H_2O	Water
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HER	Human epidermal growth factor receptor
HMW	High molecular weight
h	Hour
HRP	Horseradish peroxidase
I	Isoleucine
IL-6	Interleukin-6
IPTG	Isopropyl β-D-1-thiogalactopyranoside

K	Lysine
K_a	Association rate constant
K_d	Dissociation rate constant
K_D	Equilibrium dissociation constant
kDa	Kilodaltons
L	Leucine
LB	Lysogeny broth
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LSCM	Laser scanning confocal microscopy
LDS	Lithium dodecyl sulphate
LTQ	Linear trap quadrupole
M	Molar
MCF	Maximum clot firmness
MES	2-(*N*-morpholino)ethanesulfonic acid
Met	Methionine
mg	Milligram
mg/l	Milligram per litre
mg/ml	Milligram per millilitre
min	Minutes
ml	Millilitres
ML	Maximum lysis
mM	Millimolar
mm^2	Millimetres squared
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MW	Molecular weight
Na	Sodium
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NaH_2PO_4	Sodium phosphate monobasic
NETs	Neutrophil extracellular traps
ng	Nanograms
ng/ml	Nanograms per millilitre
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometre
nM	Nanomolar
NP	Normal pool

NTP	N-terminal peptide
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PB	Permeation buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PDB	Protein database
PEG	Polyethylene glycol
PI	Plasmin inhibitor
Plg	Plasminogen
pM	Picomolar
PPP	Platelet poor plasma
Pro	Proline
PSMA	Prostate specific membrane antigen
PVDF	Polyvinylidene difluoride
R	Arginine
RBC	Red blood cell
rFVIIa	Recombinant FVIIa
rFVIII	Recombinant FVIII
RM	Repeated measures
ROTEM	Rotational thromboelastometry
rpm	Revolutions per minute
RU	Response unit
SctPA	Single chain tPA
Sc-uPA	Single chain uPA
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
s	Seconds
SEM	Scanning electron microscopy
Serpin	Serine protease inhibitor
SH2	Src-Homology 2
SPR	Surface plasmon resonance
SQT	Stefin A quadruple mutant-Tracy

Streptokinase	SK
Sumo	Anti-SUMO Affimer
SUMO	Small ubiquitin-like modifier
T&L	Turbidity and lysis
TAFI	Thrombin activatable fibrinolysis inhibitor
TAFIa	Activated thrombin activatable fibrinolysis inhibitor
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
tctPA	Two chain tPA
tc-uPA	Two chain uPA
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Thr	Thrombin
TNC	Tenascin C
tPA	Tissue plasminogen activator
TRAP	Thrombin receptor activating peptide
TTP	Thrombotic thrombocytopenic purpura
TXA	Tranexamic acid
U/ml	Units per millilitre
µg/ml	Microgram per millilitre
µM	Micromolar
uPA	Urokinase type plasminogen activator
UPLC	Ultra-performance liquid chromatography
V	Volts
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WB	Wash buffer
WLB	Wash/load buffer

Chapter 1 Introduction

The formation of a blood clot is vital in preventing blood loss following vessel injury, however, imbalances in the formation, stability, or lysis of these clots can lead to undesired bleeding or thrombus formation. Blood clots are formed of a skeleton of fibrin fibres with platelets, red and white blood cells embedded in this network. The thrombin-catalysed conversion of soluble plasma fibrinogen into insoluble fibrin represents a crucial step in blood clot formation. Regulation of fibrin clot formation and its breakdown is achieved at multiple levels by inhibitors of coagulation and of lytic proteins. Thus, following injury, both the cellular and protein arms of coagulation are activated and combine in a complex series of reactions to form a haemostatic plug in order to stop blood loss.

Bleeding complications following traumatic vessel injury represent an important cause of morbidity and mortality, secondary to blood loss or treatment modalities aimed at replacing blood components [1, 2]. At the other end of the spectrum, vascular occlusive disease, including myocardial infarction, stroke and venous thrombosis, remain the main cause of mortality in the Western population [3]. Therefore, there is potential benefit in manipulating the fibrin network, in instances where clot lysis must be delayed, such as following acute trauma, and also when enhanced lysis of clots is advantageous, such as in occlusive vascular disease. Attempts have been made at stabilising fibrin networks following traumatic vessel injury while prevention of fibrin network formation and/or facilitation of clot lysis have been used for the treatment of acute and chronic vessel occlusion.

1.1 Coagulation

The initial response to vessel damage involves platelet activation and aggregation to form a platelet plug. Activation of coagulation in the secondary stage of haemostasis results in the formation of a network of fibrin fibres surrounding aggregated platelets, and the formation of a stable clot to seal the vessel wall.

Exposure of the subendothelium causes platelets to adhere to the site of injury via receptors on the platelet surface. Platelets bind to von Willebrand factor (vWF) and collagen via platelet receptors glycoprotein (GP)Ib-V-IX and GPVI respectively [4]. Activation of platelets occurs following adhesion to these extracellular matrix components and interaction with soluble agonists such as thrombin. Activated platelets change shape, express activated integrins, and release autocrine agents which causes further platelet adhesion and activation [5]. Platelet activation also exposes phosphatidylserine on the membrane surface which drives cell-based thrombin generation [6]. Platelets interact with fibrinogen via receptor GPIIb/IIIa, causing platelets to aggregate via fibrinogen bridges, and produce fibrin clots through the action of thrombin, which finally contract to form a tightly packed thrombus [5].

1.1.1 The coagulation cascade

The formation of a fibrin clot is the final step in the coagulation cascade, and is initiated through two pathways, the intrinsic or extrinsic pathways. The intrinsic pathway is so named because all the components are present within the blood, whereas the extrinsic pathway requires an external factor – tissue factor (TF) from the subendothelial layer of the vessel wall.

The intrinsic pathway is initiated by exposure and activation of FXII to FXIIa following contact with negatively charged surfaces including collagen, polyphosphates released from platelets and neutrophil extracellular traps (NETs) [7-10]. This generates small amounts of FXIIa, which activates prekallikrein to kallikrein. There is some reciprocal activation of FXII by kallikrein, and prekallikrein by FXIIa, resulting in a positive feedback loop. FXIIa then activates its downstream substrate FXI to FXIa. FXIa activates FIX to FIXa, allowing

formation of the intrinsic tenase complex of FIXa and FVIIIa and activation of FX to FXa [11]. The extrinsic pathway begins with TF and activated FVII, inducing sequential activation of coagulation factors, resulting in the activation of FX. The two pathways converge at the activation of FX which converts prothrombin to thrombin, and the subsequent conversion of fibrinogen to fibrin [5]. The coagulation cascade is summarised in Figure 1-1.

However, the intrinsic/extrinsic pathway is thought to be mainly an *in vitro* classification, *in vivo*, the intrinsic pathway is activated in parallel with the extrinsic pathway [5]. A newer model of coagulation describes coagulation as three phases; initiation, amplification and propagation and begins with the exposure of TF to plasma. The initiation phase starts after damage to the vessel wall, platelets adhere to the site of injury and become partially activated. Exposed TF binds FVII, promoting proteolysis and activation to FVIIa. The TF/FVIIa complex activates trace amounts of FIX and FX resulting in the generation of FIXa and FXa, respectively. FXa associates with cofactor FVa to form a prothrombinase complex on TF expressing cells, which converts prothrombin into thrombin [5].

Amplification occurs via the accumulation of thrombin generated during initiation. This thrombin activates more of the platelets at the site of injury and also converts platelet-derived FV to FVa, amplifying prothrombinase activity. Additionally, thrombin activates FVIII to FVIIIa which acts as a cofactor to FIXa on the surface of activated platelets to support FXa generation. FXI is also converted to FXIa by thrombin [5].

While the initiation phase occurs at the site of vessel injury, the propagation phase occurs on procoagulant phospholipid surfaces, such as activated platelets [5]. FXIa converts FIX into FIXa which then associates with thrombin activated FVIIIa. The tenase complex of FIXa/FVIIIa then catalyses the conversion of FX to FXa, and FXa then forms a complex with FVa to produce a sufficient amount of thrombin to form many fibrin fibres [5].

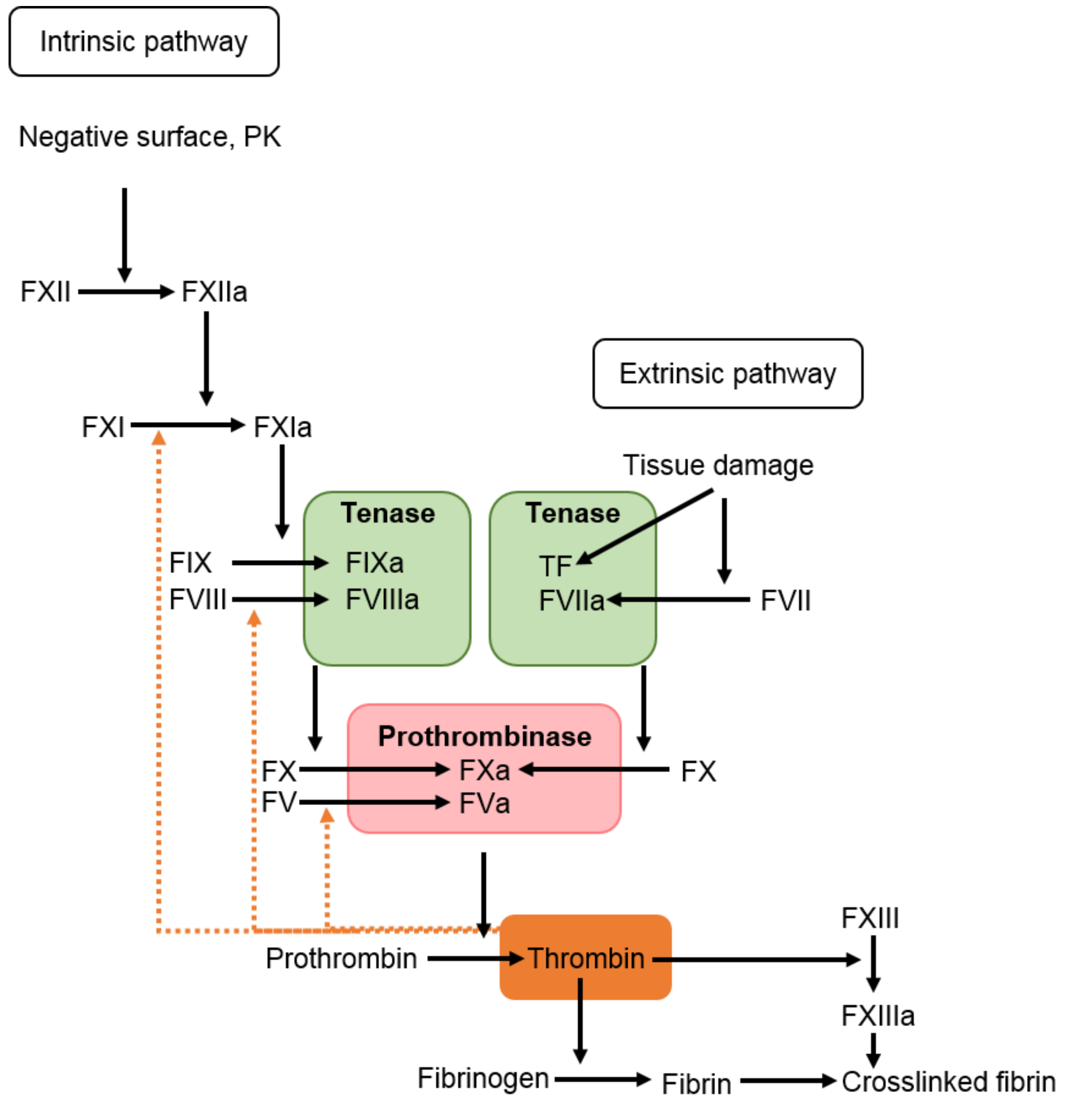


Figure 1-1 The coagulation cascade. The intrinsic pathway is initiated after activation of factor (F) XII by contact with a negatively charged surface, and involves prekaillikrein (PK). The extrinsic pathway is initiated following tissue damage and exposure of tissue factor (TF). Both pathways converge at the activation of FX to FXa and conversion of prothrombin to thrombin. Thrombin generation is further enhanced by positive feedback mechanisms (orange arrows). Thrombin cleaves fibrinogen to fibrin which polymerises to form fibrin fibres and also activates FXIII which introduces crosslinks into the fibrin network, stabilising it.

1.1.2 Fibrinogen to fibrin

Fibrinogen is a plasma glycoprotein of 340 kDa and 45 nm in length [12]. This protein is synthesised in the liver and circulates in plasma at 1.5-4.0 mg/ml but can reach higher levels in disease states [13]. Fibrinogen is an acute phase protein and its plasma levels increase rapidly under inflammatory conditions [14, 15]. Fibrinogen molecules are comprised of two sets of three polypeptide chains Aα, Bβ and γ chains held together by 29 disulphide bonds. All six chains converge at the central E region, where cleavage sites for thrombin are located. The E region is connected to two distal D regions by stretches of coiled coil consisting of Aα, Bβ and γ chains in alpha helical conformation. The Bβ and γ chains end in the D regions, whilst the C-terminal ends of the Aα chains extend out from the D regions in a coiled coil, ending in the αC globular domains [16] (Figure 1-2).

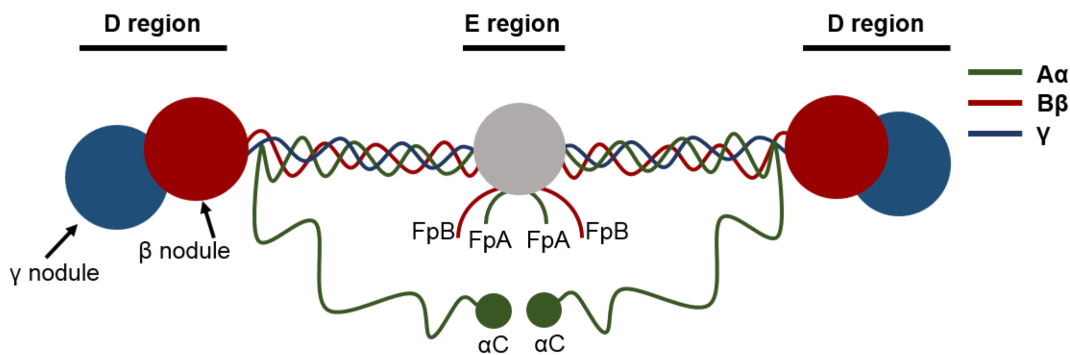


Figure 1-2 Fibrinogen protein structure. Fibrinogen consists of three pairs of polypeptide chains, Aα (green), Bβ (red) and γ (blue). The E region contains the N-termini of all six chains and is the location of cleavage sites for thrombin – fibrinopeptides (Fp) A and B. The Bβ and γ chains terminate in the D region, while the Aα chain extends beyond the D region, ending in globular αC domains.

Fibrin polymerisation is initiated upon the thrombin-catalysed removal of 2 fibrinopeptides A and 2 fibrinopeptides B from the Aα and Bβ chains respectively. The fibrinopeptides are short acidic sequences at the N-termini of the Aα and Bβ chains, removal of which exposes polymerisation sites and allows the fibrin monomers to spontaneously polymerise to form a fibrin network [12].

Thrombin first cleaves fibrinopeptide A (FpA) from the Aα chain between Arg16 and Gly17, which exposes a new N-terminal sequence Gly-Pro-Arg called “knob A” on the E region. Knob A binds specifically to a binding pocket “hole a” located

in the D region on the γ chain of a neighbouring fibrin monomer [17] (Figure 1-3A). This allows one fibrin molecule to bind two other fibrin molecules, and the formation of fibrin polymers, or protofibrils, composed of fibrin monomers overlapping in a half staggered fashion [18] (Figure 1-3B). Fibrinopeptide B (FpB), a 14 amino acid residue, is cleaved at a slower rate than FpA. Cleavage of FpB from the Bβ chain results in the exposure of a new N-terminal sequence Gly-His-Arg, "knob B" in the E region which is specific for a second binding pocket "hole b" in the D region of the β chain on a neighbouring molecule [12] (Figure 1-3C and D). The delay in FpB release means that most FpB is cleaved after the process of fibrin polymer formation has started [19]. Cleavage of FpB may release the αC domains from the E region [20], contributing to the lateral aggregation of protofibrils into fibrin fibres by allowing αC domains to interact intermolecularly [21] (Figure 1-3C).

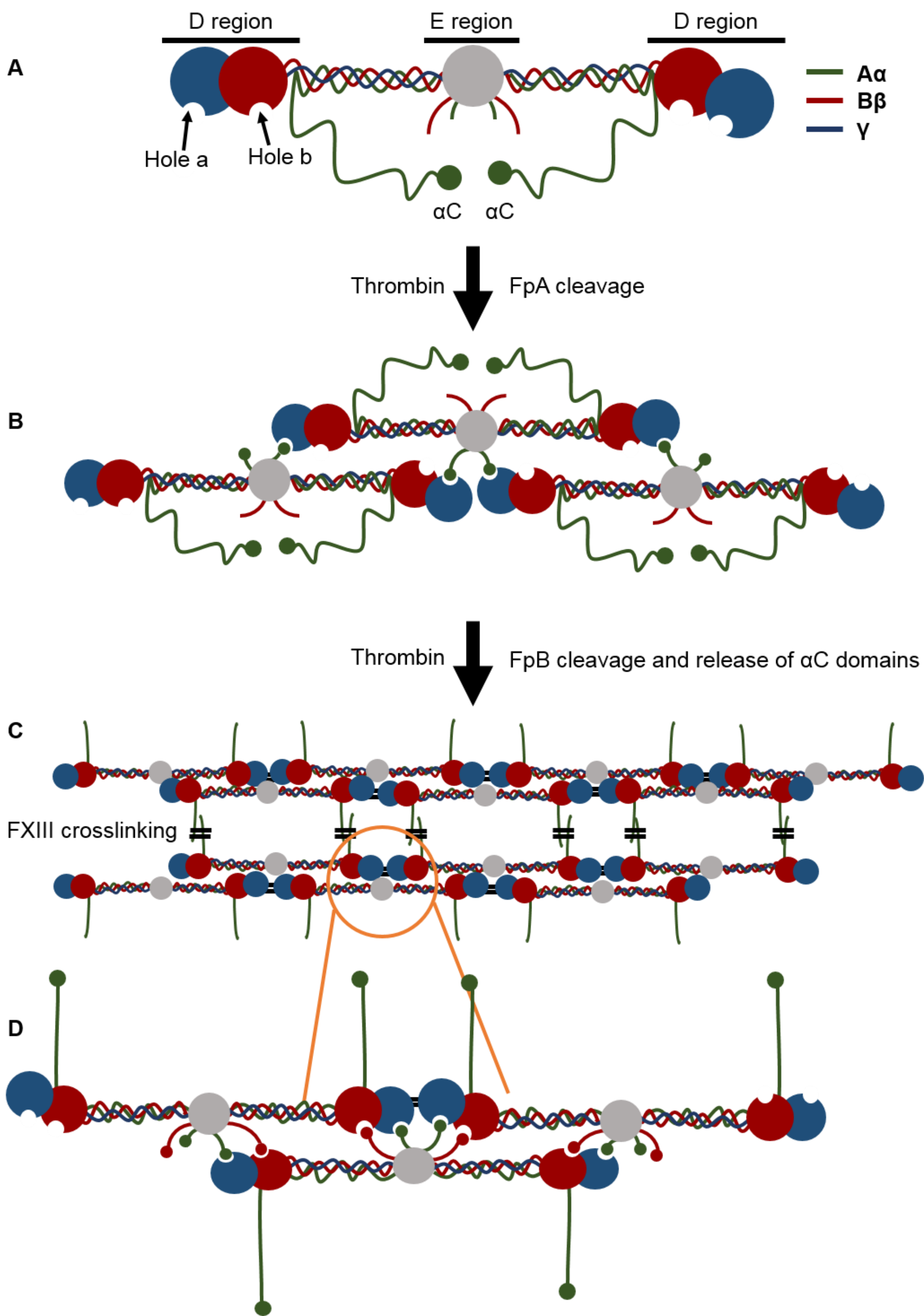


Figure 1-3 Fibrin polymerisation. A. Thrombin first cleaves fibrinopeptide A, exposing "knob A" in the E region of fibrin which is able to bind "hole A" in the D region γ nodule of a neighbouring fibrin monomer. **B.** This allows fibrin monomers to align into protofibrils. **C.** Thrombin cleaves fibrinopeptide B (FpB) at a slower rate, and this exposes "knob B" which is able to bind "hole B" in the D region β nodule of a neighbouring fibrin monomer. Cleavage of FpB also releases αC domains. Thrombin also activates FXIII which introduces crosslinks (black lines) between fibrin γ chains and α chains. **D.** Close up of the interactions between D-E-D regions in the fibrin network.

In addition to cleavage of fibrinopeptides, thrombin also activates FXIII. FXIII is a member of the transglutaminase family of enzymes, responsible for crosslinking the fibrin network, resulting in the formation of a clot which is composed of thinner fibres, has smaller pores, is more stiff, and more resistant to lysis [22-24]. Plasma FXIII is a heterotetramer of 320 kDa, consisting of 2 A subunits and 2 B subunits that are held together non-covalently [25-27]. The active site of the enzyme is found in the A subunit, while the B subunit serves as a carrier for the catalytic A subunit [25]. FXIII is also found in platelets where it exists as a dimer, formed of 2 A subunits only. Platelet FXIII also plays a role in the crosslinking of fibrin fibres [25, 28].

The activation of FXIII is a two-step process, first thrombin cleaves a 37 amino acid residue from the N-terminal of the A subunit [29], and then calcium dependent dissociation of the B subunits exposes the activated A subunits of activated FXIII (FXIIIa) [30-32]. FXIIIa stabilises the forming protofibril by introducing ε-N-(γ-glutamyl)-lysine crosslinks between residues in the fibrin γ- and α- chains. γ chain crosslinking occurs first, between Gln398 or 399 of one molecule and Lys406 on another during protofibril formation [24, 33]. α chain crosslinking occurs at a slower rate, between Gln221, Gln237, Gln328 and Gln366 and numerous lysine residues [34-36]. A small amount of α-γ chain crosslinking can also occur [37, 38].

FXIIIa also crosslinks proteins into the fibrin network, including plasmin inhibitor (PI) [39], thrombin activatable fibrinolysis inhibitor (TAFI) [40], plasminogen activator inhibitor-2 (PAI-2) [41] and complement protein C3 [42, 43], further increasing resistance of the clot to lysis.

FXIIIa-mediated crosslinking of fibrin α chains promotes the retention of red blood cells (RBCs) in clots, which consequently increases clot weight and determines clot composition [44]. RBCs are not passively trapped in the fibrin network, but must be actively retained within the thrombus by FXIII enzyme activity during clot contraction [45].

1.1.3 Clot contraction and cellular contribution to clots

Formation of a platelet plug and the conversion of fibrinogen to fibrin fibres during blood clotting stems bleeding at the site of vascular injury. Subsequently, the clot contracts through the action of cytoplasmic motility proteins inside platelets. Fibrin is important in clot contraction as it provides connections between platelets allowing the transmission of force during contraction [46, 47]. Platelet interaction with fibrinogen and fibrin is mediated through platelet integrin GPIIb/IIIa [48]. *In vivo*, thrombin induces fibrinogen binding to GPIIb/IIIa simultaneously to converting fibrinogen to fibrin. The ligand for activated platelet GPIIb/IIIa becomes polymerised fibrin, rather than fibrinogen. The platelet-fibrin meshwork formed through these interactions enables platelet mediated clot contraction [48]. The process of clot contraction aids in the restoration of haemostasis by forming a seal around the injury and helps to restore blood flow to the affected area by shrinking the clot [49].

Following clot contraction in FXIII-crosslinked clots, incorporated RBCs are compressed into polyhedracytes [47]. Increased RBC retention in clots decreases clot susceptibility to lysis [50], thought to be related to reduced diffusion of lytic enzymes into contracted clots containing tightly packed polyhedral erythrocytes [51]. Scanning electron microscopy (SEM) studies of fibrin structures formed in the presence of RBCs have demonstrated thinner fibres in RBC-rich areas, which were associated with a slower rate of plasmin digestion [50].

Platelets and RBCs are not the only cells to impact clot properties. Neutrophils within fibrin clots can release web-like bundles of genetic material upon their activation. These neutrophil extracellular traps (NETs) are composed of DNA fibres comprising histones and antimicrobial proteins [52]. Originally, NETs were described as a defence mechanism, trapping and possibly eliminating a wide variety of pathogens. However, more recent research has revealed that NETs may have a prothrombotic role, with links between NETs and arterial and venous thrombosis in animal models, as well as in humans [52]. NETs provide a backbone for RBC adhesion, as well as platelet adhesion and aggregation [53]. Activated platelets, as well as interacting with NETs, can also cause the production of NETs, in response to various platelet agonists such as collagen,

ADP and thrombin [54]. NETs support the accumulation of prothrombotic molecules like vWF and fibrinogen, supporting fibrin formation [53]. NETs, being composed of chromatin, are susceptible to degradation by DNase I, and so DNase I has been considered a potential therapeutic target to aid in the dissolution of large thrombi [55].

1.1.4 Anticoagulant pathways

Coagulation processes are tightly regulated to prevent widespread clot formation. Control is achieved through the action of anticoagulant proteins including protein C, tissue factor pathway inhibitor (TFPI) and antithrombin.

Protein C circulates as a proenzyme and is activated by thrombin bound to cofactor thrombomodulin on the surface of endothelial cells [56, 57]. Activated protein C (APC) inhibits coagulation factors FVIIIa and FVa, cofactors in the intrinsic tenase and prothrombinase complexes, respectively [58]. Protein S and FV are cofactors for APC, enhancing APC's anticoagulant activity. Cofactor Protein S is sufficient for inactivation of FVa by APC, whereas regulation of FVIIIa by APC requires both Protein S and FV [59, 60]. Protein S is also a cofactor for TFPI-dependent inhibition of FXa [61]. TFPI is a Kunitz type protease inhibitor that inhibits free FXa, and also TF-FVIIa in a FXa dependent manner [62]. Unlike TFPI, antithrombin is a serpin (serine protease inhibitor) type inhibitor. Antithrombin has high affinity for thrombin, FIXa, FXa, and is considered one of the most important inhibitors of thrombin generation. The inactivation of these proteases by antithrombin is enhanced by heparin. Long chain heparins are able to bind antithrombin, FIXa and FXa, bringing them close together and enhancing anticoagulant function [63].

1.2 Fibrinolysis

Normal physiology ensures a balance between fibrin clot formation and lysis in order to prevent widespread vascular occlusion or excessive bleeding. Plasmin is the enzyme responsible for fibrin breakdown (fibrinolysis) and is generated from plasminogen on the surface of the fibrin clot, or on cell surfaces, through the action of the serine proteases tissue plasminogen activator (tPA) or by urokinase type plasminogen activator (uPA) [64] (summarised in Figure 1-4).

1.2.1 Plasminogen activators uPA and tPA

uPA is produced by monocytes, macrophages, and urinary epithelium and has a half-life of 4-8 min in the circulation [65]. uPA consists of a C-terminal serine protease domain, a kringle domain and a growth factor domain (GFD) [66]. uPA is secreted as a single chain protein (sc-uPA) with low catalytic activity in plasma [67]. Sc-uPA is activated to two chain uPA (tc-uPA) by proteolysis of the L158-I159 bond by plasmin or kallikrein, which exposes the serine protease site [68, 69]. uPA has a low affinity for fibrin, as its kringle domain does not have a fibrin binding site [65]. However, tc-uPA rate of Glu-plasminogen activation is 10-times higher in the presence of fibrin, which may be due to conformational change of plasminogen upon binding to fibrin [70]. In contrast, sc-uPA shows more fibrin specificity than tc-uPA but has an intrinsic plasminogen activating capacity of 0.25% that of tc-uPA [71]. uPA primarily activates cell surface bound plasminogen, although it can also activate plasminogen in solution, so does not rely on a colocalisation mechanism like tPA [71]. uPA has a well-established role in extracellular matrix remodelling, growth factor activation and initiation of intracellular signalling [66], but the exact role of this protein in facilitating clot lysis *in vivo* is unclear. While uPA functions mainly in extravascular locations, tPA is the primary activator of the fibrinolysis system in circulation [64].

tPA is synthesised and released by endothelial cells, and like uPA, tPA has a short half-life of ~4 min in the circulation [65]. tPA is a 70 kDa protein which circulates in the plasma at 5 ng/ml (0.07 nM). tPA is regulated by release from the endothelium, by binding to fibrin and by binding to inhibitors, rather than zymogen activation as many of the other enzymes of the coagulation reactions [72]. tPA is secreted in an active, single chain form (sctPA), which can undergo cleavage at Arg275 by plasmin to convert it to a more active two chain form (tctPA) [73, 74]. In the absence of fibrin, conversion of sctPA to tctPA increases plasminogen activation rates from 3 to 10 fold, while in the presence of fibrin, tPA activity is increased 100-1000 fold, and sctPA and tctPA have comparable catalytic rates [65, 75].

tPA consists of a finger-like domain, an epidermal growth-factor-like (EGF) domain, two kringle domains and a serine-protease domain [76]. Interaction of tPA with fibrin is primarily through its finger domain (Lys-independent) and one of

its two kringle domains (Lys-dependent) [77, 78]. The enhanced rate of tPA-mediated plasminogen activation in the presence of fibrin is due to the formation of a ternary complex, co-localising tPA and plasminogen on the fibrin surface [74].

1.2.2 Plasminogen

Plasminogen is a 92 kDa glycoprotein present in blood plasma at a concentration of 200 μg/ml (2.2 μM) [79]. There are two forms of plasminogen; native Glu-plasminogen (which has NH_2 terminal glutamic acid) and Lys-plasminogen (NH_2 terminal lysine), the latter of which has a stronger affinity for fibrin [80]. Glu-plasminogen has seven domains; the N-terminal peptide (NTP), kringles 1-5, and a serine protease domain. Plasminogen binds to fibrin via its kringle domains [80, 81]. Plasminogen's kringle domain 1 and 4 are high and low affinity lysine binding sites, respectively [70].

An important factor in determining plasminogen activation is conformational regulation. Inter-domain bonds help maintain Glu-plasminogen in a relatively inert closed spiral structure, in which the activation cleavage site at Arg561 and the pro-activation site at Lys77 are inaccessible to plasminogen activators [82]. The exact mechanisms responsible for Lys77 becoming accessible are unknown, but it is thought that a conformational change upon fibrin binding is involved. If the NTP is cleaved at Lys77 by plasmin to yield Lys-plasminogen, the structure unfolds and Arg561 becomes accessible to plasminogen activators [82], and thus Lys-plasminogen is activated to plasmin more rapidly than Glu-plasminogen [74].

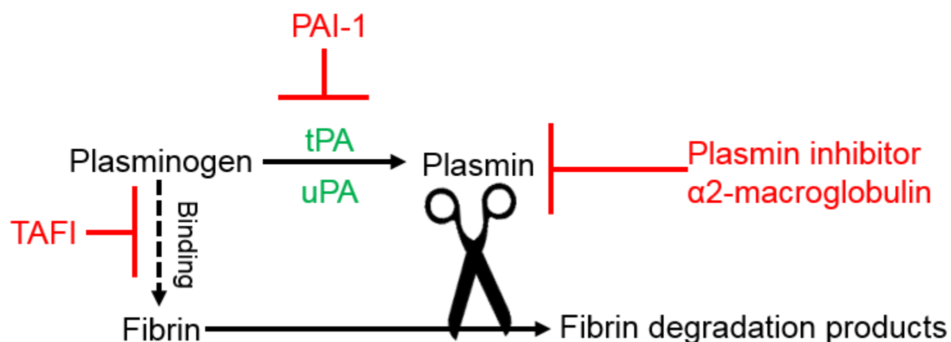


Figure 1-4 The fibrinolytic system. Fibrin clots are degraded by plasmin, which is formed by cleavage of plasminogen by tissue plasminogen activator (tPA) or urokinase type plasminogen activator (uPA). Plasmin digestion of fibrin is inhibited directly by plasmin inhibitor (PI) or to a lesser extent by α2-macroglobulin. Plasminogen activators are inhibited by plasminogen activator inhibitor 1 (PAI-1), or by thrombin activatable fibrinolysis inhibitor (TAFI) which is able to cleave C-terminal lysine residues in partially digested fibrin that are needed for plasminogen binding and activation.

1.2.3 Plasmin digestion of fibrin(ogen)

Intact fibrin has ~100 lysine residues, but initially has no C-terminal lysine residues. Exposure of cryptic tPA and plasminogen binding sites upon conversion of fibrinogen to fibrin initiates fibrinolysis [83]. Binding sites for both tPA and plasminogen have been identified at the periphery of the fibrin molecule [65].

Both tPA and plasminogen bind to the αC domain (Aα 392-610) with high affinity (K_D 33 nM and 32 nM, respectively) to sites which are cryptic in fibrinogen [84]. This binding is Lys-dependent for both tPA and plasminogen and occurs via independent sites, as saturating amounts of plasminogen do not prevent binding of tPA and vice versa [84]. Exposure of these tPA and plasminogen binding sites in the αC regions may follow the switch from inter to intramolecular interaction of the αC regions during fibrin formation [85, 86], or may require formation of αC polymers (following FXIIIa crosslinking) [35]. It has been reported that cross-linked αC structures have 2-fold higher plasmin generating activity when compared with non-cross linked αC chains [84].

There are also cryptic tPA and plasminogen binding sites located in the fibrin D region Aα chain residues 148-160 which can bind both plasminogen and tPA [87, 88] with similar affinity (K_D ~ 1 μM) [87, 89]. However, the huge molar excess of plasminogen over tPA in the blood means that under physiological conditions this binding site would be saturated with plasminogen [83]. Binding of plasminogen to this site is Lys-dependent, suggesting that interaction with fibrin is mediated by plasminogen's kringle domains [87]. There is also a tPA binding site in the D region γ chain residues 312-324 which binds tPA through tPA's finger domain (Lys-independent) [90-92]. Binding of tPA and plasminogen to fibrin D region at these two sites follows formation of D-E-D complexes during fibrin fibre assembly [89, 90]. These plasminogen and tPA binding sites in the D region (Aα 148-160 and γ 312-324) are located in close proximity to each other (within 45 Å), shorter than the length of Lys-plasminogen (140 Å) [93]. Thus, binding of tPA and plasminogen to these sites brings them close together and facilitates activation of plasminogen.

Degradation of fibrin by plasmin generates C-terminal lysine residues which provide new binding sites for plasminogen and plasmin [94]. This serves as a

positive feedback mechanism by promoting the binding and activation of more plasminogen to plasmin, promoting plasmin-mediated conversion of Glu-plasminogen to Lys-plasminogen, and by binding plasmin and protecting it from inhibition by plasmin inhibitor [95, 96]. Partially degraded fibrin (e.g. fragment X) is better at stimulating tPA-mediated Glu-plasminogen activation than intact fibrin, further enhancing fibrinolysis [97] supporting the concept that fibrinolysis is partially a self-activating process.

The sequence of fibrin digestion by plasmin is therefore as follows; the αC domains are an early target for plasmin cleavage, and peptides are cleaved from the C-terminal ends of the Aα, yielding fragment X (~260 kDa) and αC fragments (Figure 1-5A). Cleavage of the Bβ and γ chain C-termini and further digestion of the α chain generates fragment Y (~160 kDa) and fragment D1 (~100 kDa). Fragment D1 consists of the C termini of Aα, Bβ and γ chains, and it can be further digested to D2 (~97 kDa) and D3 (~87 kDa) that differ from D1 in more complete digestion of the γ chains from their C-terminal ends [98]. Digestion of the intact half of fragment Y creates fragment E (~60 kDa), a dimer containing disulphide bonded Aα, Bβ and γ chain N-termini [99]. Figure 1-5B describes the plasmin digestion of polymerised fibrin into D-E-D complexes of varying size [100-102]. Degradation of crosslinked fibrin also yields D-dimer, which is used clinically as a marker of ongoing fibrin formation and degradation [103].

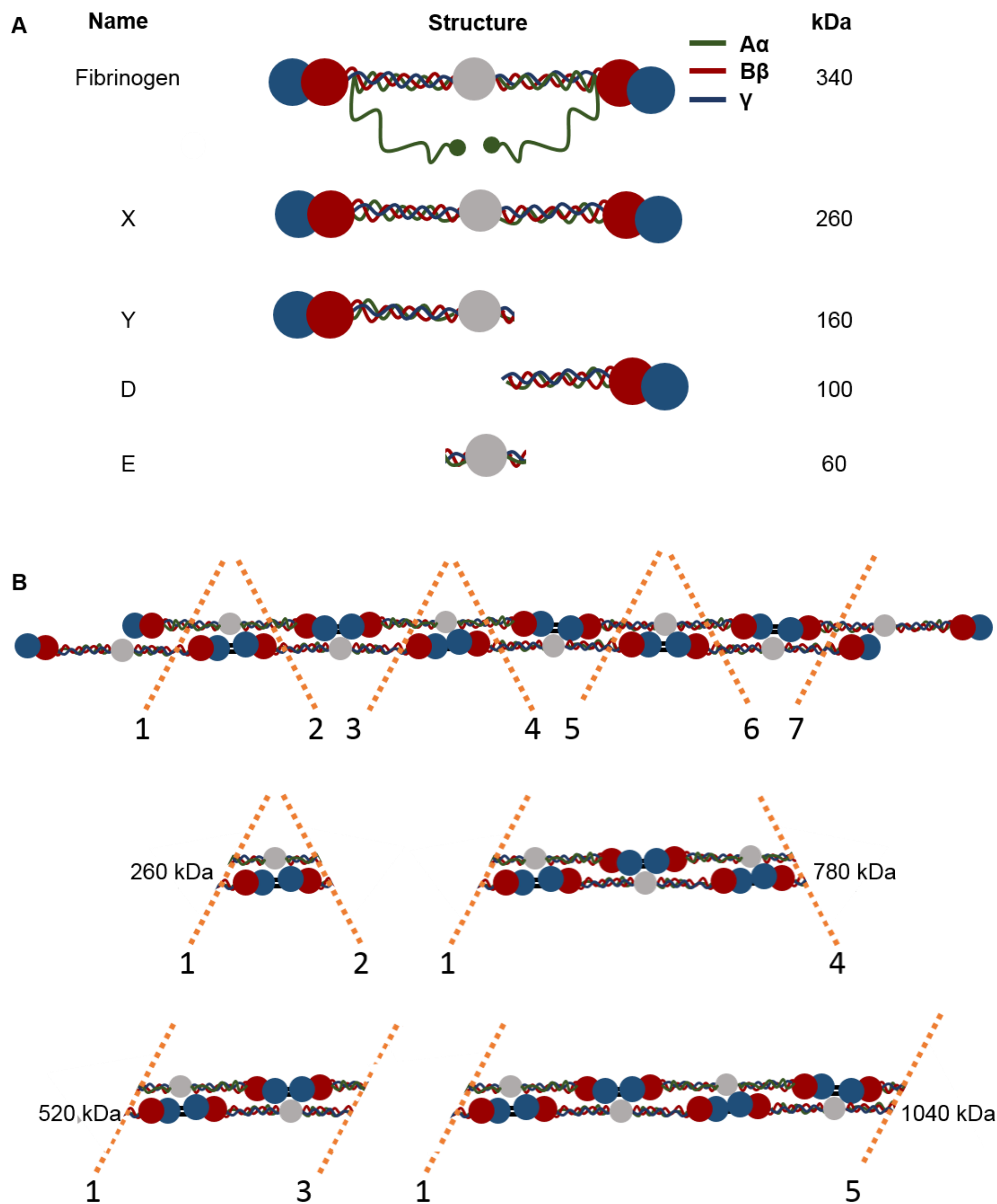


Figure 1-5 Fibrinogen and fibrin degradation products. A. Fibrinogen (340 kDa) and the products of its digestion by plasmin are illustrated, with their name and approximate molecular weight in kilodaltons (kDa). **B.** Structure of FXIIIa-crosslinked protofibril and the products of its plasmin digestion. Crosslinks between γ chains in the D regions of adjacent fibrin monomers are depicted with black lines. Fibrin is cleaved between non-covalently associated D-E-D regions as indicated with diagonal orange lines to produce fibrin degradation products. Figure adapted from Walker and Nesheim, 1999.

1.2.4 Inhibitors of lysis

Several proteins prevent unregulated plasmin or plasminogen activator activity, thereby inhibiting excessive clot lysis, these are summarised in Table 1-1.

1.2.4.1 PAI-1

Plasminogen activator inhibitor (PAI-1) is a single chain glycoprotein of 52 kDa released by endothelial cells, monocytes, macrophages, hepatocytes, adipocytes and platelets. PAI-1 is a rapidly acting physiological inhibitor of both tPA and uPA that, like other serpins, forms a stable 1:1 complex with its target proteases, inhibiting their action [104]. PAI-1 can also inhibit other serine proteases, including plasmin and APC [105]. PAI-1 is synthesised in an active form, but converts into a stable nonreactive form under normal physiological conditions [106]. Association of PAI-1 with vitronectin, a component of plasma and the extracellular matrix, stabilises the labile PAI-1 which has a half-life of ~1 h [107, 108], preventing premature lysis of a developing thrombus.

Plasma levels of circulating PAI-1 are low (~24 ng/ml, 0.46 nM) but in platelet-rich thrombi the local concentration can be high due to production by activated platelets and endothelial cells [106, 109]. PAI-1 release is stimulated by many markers of an inflammatory response, such as growth factors, cytokines and lipoproteins [70]. It has been suggested that PAI-1 has a role in cardiovascular disease (CVD), metabolic disorders and cancer. Elevated levels of PAI-1 have been associated with myocardial infarction, coronary artery diseases, venous thromboembolism, insulin resistance and obesity [105].

1.2.4.2 PAI-2

PAI-2, another member of the serpin family, is an inhibitor of fibrinolysis found in human plasma during pregnancy. PAI-2 is present as both a 47 kDa intracellular form, and a 60 kDa secreted form. PAI-2 circulates in plasma at <10 ng/ml (<0.17 nM) [106]. PAI-2 inhibits uPA, and to a lesser extent tPA [70]. PAI-2 can be crosslinked to the fibrin clot by FXIIIa to several lysine residues in the fibrin α chain [110].

1.2.4.3 Plasmin inhibitor

Plasmin inhibitor (PI, α2-antiplasmin, α2-plasmin inhibitor) also belongs to the serpin superfamily of proteins. PI is a single chain glycoprotein of 70 kDa, which is produced in the liver and circulates in plasma at a relatively high concentration (70 µg/ml, 1 µM) [105]. PI is a key protein in blood haemostasis, as evidenced by the disorders caused by homozygous deficiency or by protein over-production. Congenital deficiency of PI results in a severe bleeding disorder [111, 112], whilst elevated levels of PI are associated with an increased risk of first MI [113].

PI is present in two forms in human plasma, a 464-residue protein with methionine at the N-terminus (Met-PI) and a truncated 452-residue protein with asparagine at its N-terminus (Asn-PI). Human plasma contains around 30% Met-PI and 70% Asn-PI form. The latter is formed from Met-PI by the action of antiplasmin cleaving enzyme (APCE) and is more rapidly cross-linked into fibrin by FXIIIa [114-117]. Around 30-40% of circulating PI is also shortened at the C-terminus, in a form which is able to slowly bind plasminogen [118, 119].

Although free PI is able to inhibit plasmin, the fibrin-bound form of PI has a more significant effect on clot lysis [105]. The N-terminal end of PI (Gln2 of Asn-PI or Gln14 of Met-PI) can be crosslinked to Lys303 in the α chain of fibrin [120, 121]. While the N-terminal end of PI can bind to fibrin, the C-terminal domain has several lysine residues which bind to the kringle domains of plasmin [122].

	Molecular Weight	**Circulating plasma concentration**	**Primary protease target**
PAI-1	52 kDa	24 ng/ml, 0.46 nM	tPa and uPA
PAI-2	60 kDa (secreted form)	<10 ng/ml, <0.17 nM	As above, but to a lesser degree
Plasmin inhibitor	70 kDa	70 µg/ml, 1 µM	Plasmin
TAFI	60 kDa	4-15 µg/ml, 0.07–0.25 µM	Reduces activation of plasminogen
α2-macroglobulin	725 kDa	1.2 mg/ml, 1.7 µM	Variety of proteases, including plasmin

Table 1-1 Summary of inhibitors of fibrinolysis.

1.2.4.4 Thrombin activatable fibrinolysis inhibitor (TAFI)

TAFI is a non-serpin fibrinolysis inhibitor of 60 kDa, which circulates in plasma at a concentration of 4-15 µg/ml (0.07-0.25 µM) [123]. TAFI is produced in the liver and is present in platelets. TAFI circulates in an inactive zymogen form and is activated by thrombin or the thrombin-thrombomodulin complex, which cleave TAFI to release its activation peptide. The thrombin-thrombomodulin complex cleaves TAFI with a 1250-fold higher catalytic efficiency than with thrombin alone [124]. Plasmin has also been shown to activate TAFI, ensuring that TAFIa is generated near to the site of fibrin formation to prevent premature fibrinolysis [125]. An additional mechanism for localisation of TAFI is crosslinking by FXIII to the fibrin clot [40].

Activated TAFI (TAFIa) cleaves C-terminal lysine residues from partially degraded fibrin, which are critical for the binding of plasminogen and as a result, plasmin generation is reduced [82, 94, 126]. Removal of C-terminal lysine residues by TAFIa also removes plasmin binding sites, binding to which offers partial protection for plasmin against PI, shortening the half-life of plasmin [96]. Additionally, at high concentrations, TAFIa can directly inhibit plasmin [127].

1.2.4.5 α2-macroglobulin

α2-macroglobulin (α2M) is a non-serine protease inhibitor of plasmin synthesised by endothelial cells and macrophages, and found in platelet α-granules [128]. This large (725 kDa) tertrameric glycoprotein forms non-covalent complexes with plasmin, inhibiting its activity with about 10% of the efficiency of PI [129]. In addition to plasmin, α2M is able to inhibit virtually any protease, including trypsin, α-chymotrypsin and proteins from bacteria. The mechanism of action of α2M is unique, in that target proteases are entrapped rather than inactivated, which prevents access of substrates to the active site of the protease [128]. α2M has diverse functions owing to its ability to inhibit almost all proteases, regardless of activity and catalytic mechanism [128].

1.3 Factors affecting fibrin clot characteristics

Changes in fibrin clot structure are associated with changes in rates of fibrinolysis [130]. A study using fluorescently labelled fibres showed that thinner fibres are more likely to lyse after plasmin treatment, whereas thick fibres (>200±30 nm in

diameter) were more likely to elongate, and that lysis rates were greatly reduced in elongated fibres [131]. However, although single thick fibres are lysed at a slower rate than thin fibres, clots made up of more densely packed thin fibres tend to be lysed more slowly [132].

There are a number of factors which can modulate clot structure, some of which are discussed below, and summarised in Table 1-2.

1.3.1 Changes to coagulation factors

1.3.1.1 Fibrinogen

Changes to fibrinogen can be quantitative or qualitative. Previous studies have observed a relationship between elevated plasma levels of fibrinogen and risk of CVD [133-136]. Evidence suggests that high plasma fibrinogen levels influence clot structure, by modulating network density and rigidity through increasing fibre number and branch points. The increased risk of MI associated with elevated plasma fibrinogen levels may be attributed, in part, to the formation of stiffer clots in these individuals [137]. *In vivo* work has shown that elevated fibrinogen levels reduce time to vascular occlusion, and increase clot fibrin content, network density and resistance to fibrinolysis in a murine model [138], linking changes in fibrinogen concentration to changes in the fibrin network and increased risk of thrombosis.

In addition to fluctuating plasma levels of fibrinogen protein, fibrinogen can also undergo post translational modification, with impacts on clot formation and lysis parameters. Some of the modifications that fibrinogen can undergo include nitration [139], phosphorylation [140, 141], glycation [142, 143], oxidation [144] and homocysteinylation [145, 146]. These modifications have been linked with the formation of fibrin clots that are less susceptible to lysis and associated with disease states. In contrast, acetylation of fibrinogen, which occurs following aspirin treatment, causes formation of clots with thicker fibres, with increased porosity and that are more susceptible to plasmin digestion [147, 148].

1.3.1.2 Fibrinogen polymorphism and splice variants

Fibrinogen-γ’ arises from the altered splicing of γ chain mRNA, leading to a substitution of the C-terminal 4 amino acid (aa) residues of the γA chain with a

20 aa stretch [149, 150]. This variant exists in ~10% of fibrinogen molecules, and is mostly found in the heterodimer form γA/γ'. The C-terminal extension in γ' carries a negative charge, and contains a binding site for thrombin and FXIII [151, 152]. A study by Cooper et al. (2003) showed that rate of FpB release from γA/γ' fibrinogen was reduced, which was associated with delayed lateral aggregation of protofibrils, formation of thinner fibres with increased number of branch points and clots with reduced pore size [153]. Studies have shown that clots formed with γA/γ' are mechanically stiffer and show resistance to fibrinolysis [154, 155]. Fibrinogen γ' has been associated with thrombotic disease, but it is not clear whether γ' is a marker of CVD or a defined risk factor for CVD [156, 157].

Fibrinogen 420 (Fib420) is another fibrinogen variant that arises due to alternative splicing in mRNA transcription. Fib420 makes up 1-2% of the total fibrinogen concentration [158]. This variant has a higher molecular weight when compared to the most abundant form of fibrinogen (420 kDa vs 340 kDa) [159]. The additional molecular weight arises from 236 amino acid extensions on the fibrinogen Aα chains that are homologous to the C-termini of the Bβ and γ chains. Fib420 Aα chains each contain an additional globular domain (αE domains). These additional globular domains contain calcium binding sites that protect fibrinogen from proteolysis by plasmin [160]. The αE globular domains do not contain additional fibrin binding pockets or FXIII crosslinking sites [161].

A common fibrinogen polymorphism is an arginine to lysine substitution at residue 448 in the Bβ chain C-terminus, that has a frequency of 15%-20% in Caucasians [162]. Clots composed of Lys448 fibrinogen have thinner fibres, smaller pores, increased stiffness and are more resistant to lysis [163]. BβArg448Lys is associated with thrombotic and coronary artery disease [164, 165].

1.3.1.3 Thrombin

Fibrin fibre diameter decreases with increasing thrombin levels. Low thrombin concentrations (<1 nM, <0.1 U/ml) produce clots composed of loosely woven, thick fibres, whilst clots formed at high thrombin tend to be made up of thin, tightly packed fibrin strands which are relatively resistant to lysis [166, 167]. The concentration of thrombin observed during a coagulation reaction ranges from <1

nM to >500 nM [168], although low levels of thrombin (~2 nM) are sufficient for fibrin polymerisation [169].

Individual thin fibres are quicker to lyse than thick fibres, but clots formed of thin fibres tend to be densely packed and so harder to break down on a macroscopic level and are associated with decreased rate of plasmin digestion [132].

The effects of thrombin on fibrin clot structure go beyond the ability to influence fibre thickness and clot density. Thrombin can be bound by fibrinogen, the fibrinogen γ' variant has been shown to bind thrombin with high affinity. This binding has been associated with thrombin inhibition, by sequestering thrombin into fibrin clots and reducing circulating levels of this protein. However, fibrinogen-thrombin binding has also been shown to protect thrombin from inhibition by antithrombin and heparin, and may in fact localise thrombin to a growing clot [170].

1.3.1.4 FXIII

Crosslinking of fibrin fibres by FXIII increases resistance of a clot to lysis, in addition to the protein's ability to cross link PI, PAI-2, TAFI and C3 into the clot. Changes to FXIII protein have also been associated with changes to fibrin clot structure. A common polymorphism of FXIII results in the replacement of valine by leucine in codon 34 of the FXIII A subunit [171]. Val34Leu is located in FXIII activation peptide, three amino acids from the thrombin cleavage site. Activation of the Leu34 variant of FXIII by thrombin proceeds more rapidly than that of the Val34 variant [172]. Fibrin clots formed in the presence of Leu34 have thinner fibres, smaller pores, and clot formation time is accelerated [172, 173]. Interestingly, despite the formation of clots with characteristics similar to those associated with increased risk of thrombotic disease, the Leu34 polymorphism has been found to have either a protective effect, or no association with arterial or venous thrombotic disease [171]. Further studies are needed to determine the physiological relevance of this polymorphism.

1.3.2 Pharmacological therapies

There are a number of therapeutic treatments that result in changes to the coagulation system, in turn affecting fibrin clot structure and resistance to lysis,

which may be central to their mode of action. This has been reviewed extensively elsewhere [174-176].

Aspirin, an antiplatelet agent, is able to modify the fibrin network by acetylating lysine residues on fibrinogen. As mentioned in 1.3.1.1, acetylation results in an altered fibrin network which is easier to lyse [148]. Similarly, fibrin networks formed in the presence of antihyperglcyemic drug metformin have reduced FXIII crosslinking and lyse more quickly. The presence of the drug during fibrin clot formation reduced thrombin cleavage of FXIII activation peptide, in addition to thrombin cleavage of fibrinopeptides from fibrinogen, causing formation of clots with thinner fibres [177]. Work by Standeven et al. (2002) indicated that part of the cardioprotective effect of metformin may be through the drug's alterations to fibrin network characteristics [177].

A number of agents used in thrombotic disorders, including angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, β-blockers and fibrates have not been directly investigated for an effect on clot structure, but are associated with decreases in fibrinogen and PAI-1 levels. Decreases in these coagulation factors are likely to contribute to changes in fibrin network characteristics and the beneficial effects of these agents (reviewed in [174]). Factors affecting fibrin clot characteristics are summarised in Table 1-2. Pharmacological therapies targeting the fibrin network are discussed in detail in section 1.5.

Modification	Functional effects	References
Fibrinogen concentration	Increased [fibrinogen]: ↑Fibrin clot content, ↑clot density ↑resistance to fibrinolysis	[137], [138]
Fibrinogen nitration	Modification of Bβ chain C-terminal tyrosine residues 292 and 422. ↑rate of clot formation, ↑clot stiffness, ↑resistance to fibrinolysis	[139], [178]
Fibrinogen acetylation	Acetylation of lysine residues – multiple Aα residues, one residue on the Bβ chain, and two residues on the γ chain, ↓clot density, ↑clot porosity, ↑fibre diameter, ↑fibrinolysis	[179], [147], [148]
Fibrinogen phosphorylation	Modification of Ser3 and Ser345 of the Aα chain, ↑thrombin binding, ↑resistance to fibrinolysis	[180], [181], [182], [140, 141]
Fibrinogen glycation	Modification of lysine residues, ↓binding of tPA and plasminogen to fibrin, ↑clot density, ↑resistance to fibrinolysis	[179], [183], [142], [143], [184]
Fibrinogen oxidation	↑Clot density, ↓clot permeability, ↓fibre diameter, ↓tPA-mediated plasminogen activation, ↑resistance to fibrinolysis, ↑rate of fibrin formation, ↑resistance to fibrinolysis	[185], [144], [186], [187]
Fibrinogen homocysteinylation	Modification of lysine residues, ↓clot permeability, ↓fibre length, ↑fibre branching	[145], [146]
Fibrinogen polymorphisms and splice variants	Fibrinogen γ', substitution of C terminal 4 amino acid residues of the yA chain with a 20aa stretch, in γ': ↑clot stiffness, ↓rate of FpB release, ↓fibre diameter, ↓clot pore size, ↑fibrin branching, ↑resistance to fibrinolysis	[153], [154], [155]
	Fibrinogen 420 has addition globular domains on the Aα chains, containing calcium binding sites which protect fibrinogen from plasmin digestion	[160]
	BβArg448Lys polymorphism in the Bβ chain C terminus, Lys448 fibrinogen: ↓fibre diameter, ↓clot pore size, ↑clot stiffness, ↑resistance to fibrinolysis	[163]
Thrombin concentration	Elevated [thrombin]: ↓Fibre diameter, ↑clot density, ↑resistance to fibrinolysis	[166], [167]
FXIII polymorphism	Val34Leu, 34Leu variant: ↑rate of activation by thrombin, ↑rate of clot formation, ↓ fibre diameter, ↓clot pore size	[171], [172], [173]
Pharmacological therapies	Aspirin is able to acetylate lysine residues on fibrinogen, see above Metformin, ↓FXIII crosslinking, ↑rate of fibrinolysis A number of agents used in thrombotic disorders affect fibrin clot structure/lysis, although not necessarily directly. For example, reduction of PAI-1 and fibrinogen levels by angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, β-blockers and fibrates	Pharmacological therapies reviewed in [174]

Table 1-2 Factors affecting fibrin clot structure and lysis. Modifications to coagulation factors, both qualitative and quantitative can affect fibrin clot properties and resistance of a clot to plasmin digestion.

1.4 Pathological relevance of clot characteristics

Changes to clotting factors can result in lysis which is excessive (hyperfibrinolysis), or insufficient (hypofibrinolysis). There are numerous causes of both hyper- and hypo-fibrinolysis, and I will focus on those which directly arise as a result of alterations in the fibrin network.

Causes of hyperfibrinolysis can be inherited or acquired and the result of inadequate pro-coagulant mechanisms or enhanced fibrinolysis. Conversely, hypofibrinolysis occurs in conditions characterised by formation of clots which are less susceptible to degradation, and/or due to insufficient production of enzymes required for clot lysis or overproduction of anti-fibrinolytic proteins.

1.4.1 Hyperfibrinolysis

1.4.1.1 Haemophilia

Haemophilia A, B and C are rare congenital deficiencies of coagulation factors VIII, IX and XI, respectively. Bleeding episodes are common in individuals with haemophilia, some of which are spontaneous and unprovoked. Coagulation factors that are deficient in haemophilia are involved in the positive feedback mechanisms of the coagulation cascade mediated by the intrinsic tenase complex [188]. As a result of their deficiency, there is a delay in thrombin generation in the propagation phase of coagulation, while initial thrombin generation is not significantly affected [189]. In haemophilic blood, sufficient fibrin for clot formation is generated at the start of clotting, suggesting that bleeding is due to clot instability rather than fibrin deficiency [188], a theory supported by the elevated levels of D-dimer in the circulation of patients with haemophilia [190, 191]. Considering the effect that thrombin concentration has on fibrin clot structure (mentioned previously in section 1.3.1.3), fibrin clots formed in haemophilic plasma are made up of more loosely woven thicker fibres, forming clots with increased permeability that are more susceptible to lysis [192-194]. Reduced thrombin generation in individuals with haemophilia leads to a delay in TAFI generation and FXIII activation, which also contributes to clot instability [195-197].

1.4.1.2 FXIII deficiency

Crosslinking of fibrin chains by FXIIIa increases resistance to lysis by formation of γ-chain dimers and high molecular weight α-polymers, which increase rigidity and strength of the clot, preventing it from shear stress in the circulation [198]. FXIIIa also incorporates PI into the clot, preventing premature clot dissolution by the fibrinolytic system [120]. FXIII deficiency is a rare hereditary bleeding disorder, its symptoms range from mild skin bleeding, to life threatening haemorrhage such as intracranial haemorrhage [199]. Plasma concentration of FXIII correlates well with severity of bleeding [200].

1.4.1.3 Dysfibrinogenemia

Congenital defects in fibrinogen can result in absent (afibrinogenemia) or low (hypofibrinogenemia) plasma fibrinogen antigen levels or low functional activity of fibrinogen which is either at a normal (dysfibrinogenemia) or reduced (hypodysfibrinogenemia) antigen level [201].

There are more than 100 causative mutations in congenital dysfibrinogenemia. Most mutations identified are missense mutations in the N-terminal portion of the Aα-chain or in the C-terminal region of the γ-chain. These mutations result in impaired interaction between D-D regions, or D-E-D complexes, thereby affecting fibrin assembly during the early stages of clot formation [202].

Individuals with congenital dysfibrinogenemia present with highly heterogeneous phenotype, from being asymptomatic to exhibiting mild bleeding and/or thrombosis [201]. A report by Casini et al. (2016) found an association between fibrinogen variants and clinical presentation, with dense and hypofibrinolytic clots shown in thrombotic disorders while loose clots with increased permeability dominated in bleeding conditions [203].

1.4.2 Hypofibrinolysis

Hypofibrinolysis results in impaired clot dissolution and is a cause of thrombosis in multiple disease states [204]. Hypofibrinolysis may result from formation of a fibrin clot with a structure which resists lysis and/or is due to impairment in the fibrinolytic system. There is an association between fibrin clot structure and CVD, compact fibrin networks with densely packed thin fibres are associated with

increased risk of cardiovascular events [205-210]. Changes to clot structure are observed in individuals with both arterial and venous thrombotic diseases. The delay in fibrinolysis rates in dense clots formed of thin fibres may be due to reduced permeation of fibrinolytic enzymes into these clots [211], as well as reduced binding of tPA and plasminogen to densely packed, thin fibres [212].

Fibrinogen Dusart is an example of a condition in which changes to fibrin clot structure and lysis are directly related to clinical outcome, as it is associated with recurrent thrombosis [213]. Fibrinogen Dusart arises from a single base change in the Aα chain gene, which results in the amino acid substitution Aα Arg554 → Cys [214].

Fibrinogen Dusart molecules can be disulphide linked to albumin, resulting in the formation of fibrinogen-albumin complexes in affected individuals [214, 215]. Collet et al. (1996) demonstrated that the albumin-bound αC domains of Dusart fibrinogen are free-swimming rather than associated with the central E domain, and that they do not participate in intermolecular interactions [216]. This gives rise to formation of clots with thinner fibres due to impaired lateral aggregation. Clots are also more dense and have greater resistance to fibrinolysis [213] as a result of reduced binding of plasminogen to fibrin [217], and impaired plasminogen activation by tPA [218]. The disulphide bond between Dusart fibrinogen and albumin may be responsible for steric hindrance of plasminogen and tPA binding to their binding sites in the αC domain [84, 213].

1.5 Clinical targeting of the fibrin clot to modulate fibrinolysis

1.5.1 Fibrinolysis in thrombosis

Formation of an obstructive vascular thrombus can lead to myocardial infarction, stroke, and ischemic limb damage. The aim of treatment is to start reperfusion and regain blood flow to the affected vessels. Following an acute thrombotic event, surgical approaches include angioplasty and thrombectomy, while thrombolytic drugs continue to be the mainstay of medical therapy. The aim of thrombolytic drugs is to aid breakdown of obstructive thrombi by activating plasminogen [219].

1.5.1.1 Thrombolytic drugs in current use

There are a variety of thrombolytic drugs which function by enhancing generation of plasmin, including uPA, tPA and streptokinase. Streptokinase was the first enzyme to be used as a thrombolytic agent and it is still used in some countries due to its low cost, however, it is not fibrin-selective and is therefore associated with generalised proteolysis [220]. Uncontrolled plasmin generation is known to cause depletion of coagulation proteins fibrinogen, FV, FVIII and PI, promoting bleeding [221].

Although tPA is fibrin specific (in that its activity increases up to 1000 fold in the presence of fibrin), continuous infusion of the protein administered to compensate for the protein's short half-life (4 min) removes its fibrin-specificity. The result is systemic plasmin generation and bleeding complications in a large patient population [221]. Development of newer thrombolytics, with modifications that aim to improve half-life, activity, and fibrin specificity of the protein have reduced some of the problems associated with earlier drugs, e.g. second and third (e.g. Tenecteplase) generation thrombolytic drugs [221]. Tenecteplase is a mutated variant of tPA that has an extended half-life and a fibrin specific mode of action [222]. As a consequence of Tenecteplase's 14-fold enhanced relative fibrin specificity compared to tPA, there is reduced systemic plasmin generation when compared with native tPA [223].

Other approaches at achieving fibrin specificity in plasminogen activators have shown success in *in vitro* assays but none have proceeded to clinical trials. One such approach involves fusion of plasminogen activator proteins to anti-fibrin antibodies [224]. Antibody-conjugated thrombolytics have not yet made it to clinic, perhaps because of the complexity involved in their commercial manufacturing and concerns about the long-term stability of such agents [221]. Novel delivery methods for thrombolytic drugs to ensure fibrin specificity have also been the focus of much research [225]. These include encapsulation of tPA in liposomes [226], fibrinolytic-bearing erythrocytes [227], and fibrinolytic-bearing nanoparticles [228].

1.5.1.2 Alternative targets in thrombosis

One difficulty with traditional thrombolytic agents (streptokinase, tPA) is the high risk of bleeding given the general mode of action. Therefore, there is a focus to inhibit one, or a limited number of, pathway(s) in fibrinolysis in the hope that this facilitates clot lysis without significantly increasing bleeding risk. The main anti-fibrinolytic proteins of the coagulation system including TAFI, PAI-1 and PI have been explored as potential targets to inhibit for the purpose of facilitating clot lysis.

Inhibition of TAFI has been considered as a therapeutic strategy in thrombotic disorders but only a limited number of drug candidates have made it to clinical trials, which were then discontinued [229, 230]. One anti-TAFI approach by Buelens et al. (2010) saw the creation of a panel of inhibitory Nanobodies effective against the various modes of TAFI activation and activity. Nanobodies are single domain antibodies from the sera of members of the *Camilidae* family which have advantageous properties such as low immunogenicity and high affinity, solubility and stability [231]. Inhibiting PAI-1 is also a therapeutic target with anti-thrombotic potential, and the use of Nanobodies for this inhibition has been investigated [232]. These inhibitory Nanobodies showed profibrinolytic activity in an *in vitro* clot lysis assay [232], but further *in vitro* and *in vivo* characterisation is awaiting. There have also been numerous attempts at inhibiting PAI-1 with antibodies [230, 233] but none have been taken forward to the clinical arena, due, at least in some cases, to off target toxic effects. Recent work in mouse models of thrombotic stroke have shown that simultaneous inhibition of PAI-1 and TAFI with a bispecific antibody resulted in a significant enhancement of fibrinolysis in mice, without increased bleeding [234]. PAI-1's relatively short half-life of ~1 h under physiological conditions [107, 108] could limit its use as a therapeutic target, however, the counter-argument is that the short half-life makes it a suitable target for acute vascular thrombosis. Despite investigation of several PAI-1 inhibitors *in vivo* and *in vitro* to date there are no PAI-1 inhibitors clinically available [235]. One explanation for this lack of progress could be the unique structural properties of PAI-1. Crystallographic data of PAI-1 in complex with vitronectin is still incomplete and thus hampers rational design of small molecules able to bind and inactivate PAI-1 bound to vitronectin. Additionally, the mechanism by which active PAI-1 transitions to its latent form is not fully understood. Although numerous PAI-1 inhibitory compounds have been

investigated, for many, their mechanism of action remains elusive, providing yet another obstacle in the clinical application of PAI-1 inhibitors.

Numerous attempts at targeting plasmin inhibitor have been made, including the use of antibodies and mutant forms of the protein. Incorporation of PI into fibrin clots increases clot resistance to lysis [120], and so PI, particularly clot-bound PI is a potential therapeutic target. Early work by Kumada et al. (1984) reported that repeated injection of polyclonal anti-PI $F(ab')_2$ fragments reduced circulating PI levels and led to an acceleration of thrombolysis by enhancing fibrinolytic activity [236]. A more targeted approach used an antibody specific for clot-bound PI, which enhanced lysis of a human clot in a rabbit jugular vein thrombosis model [237]. Utilising knowledge of PI mechanism of action, one group mutated PI active site residue Arg364 [238] and generated an Arg-Ala mutant [239]. Both of these modified PI variants retained their ability to be crosslinked to the fibrin network by FXIIIa, but lost their plasmin-inhibitory ability, thereby enhancing fibrinolysis.

The N-terminal region of PI is crucial for the protein's cross-linking to fibrinogen [240, 241]. Kimura et al. (1985) created a 12 residue synthetic N-terminal peptide of PI that was able to reduce incorporation of native PI into fibrin networks by FXIIIa *in vitro*. Cross-linking of the synthetic peptide to fibrin accelerated spontaneous as well as tPA-induced fibrinolysis [242]. Despite promising preliminary data, none of these approaches to PI-related therapeutics have been clinically adopted, indicating that translating *in vitro* findings, and even animal work, into application in man is difficult and more complex than initially envisaged.

More recently, work by our group has shown that complement protein C3 interaction with fibrinogen can be targeted with Affimer proteins [243], which were able to abolish C3-induced prolongation of fibrin clot lysis. The role of C3 in fibrinolysis has yet to be widely recognised as an antithrombotic therapeutic target, however, two independent studies have demonstrated the presence of C3 and its metabolites in plasma clots [42, 244]. C3 binding/cross-linking into the fibrin network increases resistance of the clot to lysis [42] and it appears that the anti-fibrinolytic effects of C3 are exaggerated in some high vascular risk conditions such as diabetes [245-247]. It remains to be seen whether this approach proves to be suitable for future clinical application.

1.5.2 Fibrinolysis in bleeding

Reducing blood loss is a key goal in the setting of trauma, following surgery, and in individuals with haematological disorders. There are various strategies to this end; anti-fibrinolytic drugs aim to reduce plasmin cleavage of fibrin, either by reducing the generation of plasmin, or by targeting plasmin directly. Fibrin sealants are a relatively new class of agents that aim to minimise blood loss by facilitating the formation of a fibrin network that is resistant to lysis. In patients with haemophilia, treatments that replace the missing coagulation factor help to restore normal haemostatic function, but there are many studies to show that therapeutic strategies which stabilise the fibrin network could be beneficial when used in place of, or alongside factor replacement.

1.5.2.1 Stabilising the fibrin network in haemophilia

Standard treatment in individuals with haemophilia is focused on replacement of the missing factor to prevent or treat bleeding. In the case of individuals who develop inhibitory antibodies against the replacement factor, agents which bypass the missing factor are needed to promote clot formation. An alternative strategy being explored more recently is to decrease endogenous anticoagulant activities, such as TFPI [248], antithrombin [249], and APC [250]. I will focus here on discussing strategies that target the fibrin network.

As described previously, there is some evidence to suggest that the initial thrombin generated in individuals with haemophilia is sufficient to allow formation of a fibrin clot, but that the absence of a thrombin burst (and subsequent activation of TAFI and FXIII) means that the clot formed is unstable, and not protected from premature lysis. Thus, strategies which stabilise the fibrin network in haemophilia may allow the maintenance of the clot formed in these individuals, reducing the need for frequent factor replacement therapy. Various agents have been investigated for their potential to stabilise the fibrin network, including FXIII, thrombomodulin and TXA.

1.5.2.1.1 FXIII

FXIII has been used clinically when bypassing agents were ineffective in an individual with haemophilia. Following poor clinical response to recombinant FVIIa (rFVIIa) and activated prothrombin complex concentrate (aPCC), FXIII treatment was shown to improve clot formation time and normalised maximum amplitude in whole blood thromboelastography [251].

To explore the mechanisms by which FXIII improves the stability of haemophilic clots, a study by Beckman et al. (2018) used FVIII deficient plasma and whole blood clotted with haemostatic agents FVIII, rFVIIa, anti-inhibitor coagulation complex (factor eight inhibitor bypassing activity, FEIBA) and/or FXIII. It was found that compared to FVIII treated haemophilic plasma, FVIII + FXIII co-treatment accelerated FXIIIa formation, an effect seen without any increase in thrombin generation. Fibrin crosslinking, and PI crosslinking into the fibrin network was found enhanced following FXIII co-treatment [252]. Additionally, in the presence of FVIII inhibitors, co-treatment with FXIII (compared with haemostatic agents alone) increased whole blood clot weight. Increased RBC content of haemophilic clots may further enhance clot stability as RBCs decrease clot permeability and increase clot resistance to lysis. These data explain the mechanistics of the benefits of FXIII co-treatment in individuals with haemophilia [252].

Supra-physiological concentrations of FXIII (in the absence of other haemostatic agents) have been shown to stabilise clots made from haemophilic blood. FXIII alone was able to cause reduced fibrin clot permeability, by decreasing fibre thickness and pore size, and was effective at both low and high FVIII levels [253]. Given the cost of prophylactic factor replacement, and the fact that at least 50% of compliant patients continue to experience bleeding events [254] FXIII may be a useful clot-stabilising adjunct with clinical benefit in a factor-sparing regime. It has been speculated that FXIII treatment alone may be sufficient to maintain haemostasis in individuals with mild or moderate haemophilia [253].

1.5.2.1.2 Thrombomodulin/TAFI

TAFI is activated by thrombin, or the thrombin-thrombomodulin complex, and once activated has anti-fibrinolytic activity. TAFI activation is decreased in

haemophilia because thrombin generation through the intrinsic feedback loop is non-functional. Defective TAFI activation in individuals with haemophilia has been associated with enhanced fibrinolysis [196, 255].

Work by Foley et al. (2012) has shown that a soluble form of thrombomodulin, called Solulin, can partially correct the enhanced lysis seen in FVIII-deficient plasma through a TAFIa-dependent mechanism [256]. Blood from haemophilia patients showed increased maximum clot firmness (MCF) and prolonged clot lysis time when treated with Solulin compared to non-treated blood. [256]. Addition of TAFI directly to haemophilic plasma has also been shown to prolong lysis times and restore clot stability [255]. Importantly, Mosnier et al. (2001) showed that addition of TAFI to the plasma of haemophilia A patients with inhibitors was able to increase clot lysis times similarly to the increase observed in haemophilia patients without inhibitors [255].

1.5.2.1.3 Tranexamic acid

Stabilisation of the fibrin clot in certain populations of haemophilia patients is achieved through the systemic and local administration of anti-fibrinolytics epsilon amino caproic acid (EACA) and TXA [257, 258]. An early clinical trial in patients with haemophilia investigated the benefits of TXA as monotherapy and showed that it did not reduce the need for FVIII replacement, and did not significantly reduce major joint bleeds [259]. However, combined therapy (factor replacement + TXA) is commonly the standard of care for bleeding and prophylaxis in haemophilia patients undergoing surgery [260] and there is evidence that anti-fibrinolytic EACA is similarly beneficial as an adjunctive [261].

To explore the mechanisms of the benefits of adjunctive TXA therapy, Hvas et al. (2007) showed that a combination of recombinant FVIII (rFVIII) and TXA improved blood clotting parameters as assessed by rotational thromboelastometry (ROTEM), when compared with rFVIII treatment alone. In this study, blood samples from individuals with haemophilia A were taken before and after administration of rFVIII, and then again after administration of TXA, and whole blood clotting profiles determined by thromboelastometry. Maximum clot firmness and area under the elasticity curve were increased with rFVIII, and further increased in samples containing rFVIII and TXA [262].

In a small study by Holmström et al. (2012), combined treatment with aPCC and TXA was effective at managing bleeding episodes and in preventing haemorrhage during surgery in patients with FVIII inhibitors. Similarly to the study by Hvas et al. (2007), administration of TXA increased clot stability as measured by maximum clot firmness in ROTEM assays. Thrombin generation assays showed a shorter lag time and a higher endogenous thrombin potential after aPCC and TXA administration when compared with patient's baseline measures. Importantly, measures of thrombin generation did not exceed the values of healthy controls, suggesting there was no hypercoagulability in these patients following treatment [263].

1.5.2.2 Stabilising the fibrin network following surgery or trauma

1.5.2.2.1 TXA and EACA

TXA and EACA are lysine analogues that reversibly bind to plasminogen, inhibiting its interaction with fibrin and its activation to plasmin [264]. TXA is used therapeutically in a variety of settings, including menorrhagia, in cardiac, orthopaedic, cranial, spinal, hepatic, and pancreatic surgery. TXA can also be administered orally, topically, or intravenously for dental extractions [265, 266]. TXA is also effective at preventing death due to bleeding in trauma, when administered within 3 h [267]. Dosage of TXA for treatment of any disorder has to be considered carefully, as TXA is able to cross the blood brain barrier and increase risk of seizures [268]. However, TXA is generally considered a well-tolerated drug with few adverse effects [266]. TXA demonstrates around 6-10 fold higher affinity for lysine binding sites than EACA and is more widely used [266].

However, TXA can be profibrinolytic at high concentrations, by binding to the low affinity kringle of plasminogen and inducing a conformational change to a form which is more readily activatable by uPA (but not tPA) [94]. Additionally, TXA binding to plasmin is able to protect plasmin from inhibition by PI [188].

1.5.2.2.2 Aprotinin

Aprotinin is a direct plasmin inhibitor but the use of this agent has been associated with increased mortality and is thus no longer available in most parts of the world [269].

1.5.2.2.3 Fibrin sealants

A number of fibrin sealants have been developed which aim to reduce blood loss following surgery. Fibrin sealants are usually composed of a mixture of proteins including fibrinogen, thrombin, FXIII and anti-fibrinolytic agents. In the past bovine thrombin was commonly used, but current agents use human thrombin and fibrinogen from pooled donor plasma. The mix of proteins in a fibrin sealant ensures formation of a fibrin clot which is resistant to lysis, thus minimising blood loss [270-273]. Fibrin sealants work through multiple modes; being capable of causing blood to clot, creating a sealing barrier, and gluing tissues together [271-274]. Fibrin sealants are available in liquid, as well as patch forms. Fibrin sealants have been used in a variety of surgical procedures, including cardiac, thoracic, and orthopaedic surgery [273, 275-277].

Fibrin sealants currently available have a number of limitations (reviewed in [274]). Time taken to prepare sealants is potentially problematic, as the need for thawing the product or mixing is not ideal in emergency situations. There is a risk of thrombosis if the sealant is inadvertently injected intravascularly, due to the presence of thrombin. Also, the means of application of a fibrin sealant can present risk, as in the use of gas-driven spray devices that carry the potential risk of air emboli. Additionally, the application of too much fibrin sealant can contribute to infection and reduced healing. Anaphylactic reaction can be a side effect, due to the presence of aprotinin in selected fibrin sealants. Human-derived proteins have also been reported to cause allergy, anaphylaxis or infection [274]. Fibrin sealants made from recombinant proteins to combat this have been described but are yet to become widely available [278].

1.5.2.2.4 A novel approach to limit blood loss

An alternative approach to stemming bleeding following trauma is an engineered haemostatic polymer (PolySTAT) [279]. This polymer consists of multiple fibrin-binding domains on a linear water soluble backbone. Importantly, PolySTAT compound is specific for fibrin, and thus becomes targeted to sites of injury with no binding of soluble fibrinogen in circulation. PolySTAT functions by causing formation of non-covalent bonds between adjacent fibrin monomers, acting similarly to FXIII in crosslinking of fibres. However, clots formed in the presence of PolySTAT have altered structural properties. Compared to FXIII-crosslinked

clots, PolySTAT induced formation of thicker fibrin fibres and reduced clot permeability. SEM images of clots formed in the presence of the synthetic polymer revealed formation of mesh-like structures within fibrin clots, which, can be argued, are not physiological [279].

Addition of the polymer to whole blood in thromboelastography studies demonstrated accelerated clotting, increased clot strength and decreased clot susceptibility to lysis in the presence of PolySTAT. Furthermore, in a rat femoral artery injury model intravenous administration of PolySTAT increased survival rate in rats and decreased blood loss [280]. Further animal studies undertaken by this group showed that commercially available chitosan gauzes used to limit bleeding were more effective when impregnated with PolySTAT compound and thus that this polymer is effective *in vivo* when applied intravenously or topically [280]. Despite reduced blood loss in *in vivo* studies by this group, survival of rats was not significantly improved when comparing chitosan with and without PolySTAT [280]. Larger studies are required, perhaps with large animal bleeding models to further explore this reagent.

1.6 Engineered protein scaffolds

Antibodies are the most commonly used binding proteins with many candidates in clinical development [281] and remain important in scientific research, diagnostics and therapy. However, antibodies have a number of limitations including sensitivity to elevated temperatures and expensive production costs. Antibodies are relatively large proteins requiring glycosylation and disulphide bonds for function and stability. Production is therefore confined to expression in eukaryotic systems which are more costly than bacterial systems, and the presence of glycosylation increases batch to batch variation [282, 283]. The development of engineered protein scaffolds as an alternative to antibodies has resulted in over 50 novel non-antibody protein scaffolds. These include Anticalins [284], Affibodies [285], Ankyrins [286], engineered Kunitz domains [287], Nanobodies [288] and Affimers [289].

Small engineered protein scaffolds are associated with some general advantages over antibodies [283]. Many non-antibody scaffolds do not contain cysteine residues, allowing easier large scale production of the proteins and also allowing the addition of a free thiol to the scaffold for site-specific modification. Additionally, in general, most engineered protein scaffolds are characterised by high thermal and chemical stability, making them preferable to antibodies in applications involving exposure to harsh chemical or environmental conditions [283]. Non-antibody protein scaffolds also have some advantages over antibodies in specific applications such as *in vivo* imaging [290] (discussed further below). Details of selected engineered protein scaffolds are summarised in Table 1-3.

Engineered protein scaffold	Size	Scaffold based on	Example targets and binding affinities
Anticalins	~20 kDa	Lipocalins	- Oncogene MET, for use as imaging agents in positron emission tomography (PET), 600 pM binding affinity [291] - Prostate specific membrane antigen (PMSA), 500 pM [292] - Vascular endothelial growth factor A (VEGF-A), 25 pM [293] Anticalin-based therapeutics have demonstrated safety and tolerability in early clinical studies [294]
Affibodies	6-7 kDa	Z-domain of staphylococcal protein A	- Human epidermal growth factor receptor-2 (HER-2), 22 pM [295] - Tumour necrosis factor-α (TNF-α), 500 pM Affibodies have reached clinical development for *in vivo* tumour imaging and for inflammation disorders [296]
Affimers	~13 kDa	Type I Affimer scaffold based on human Stefin A Type II Affimer scaffold based on phytocystatin consensus sequence	- VEGF-2, ~40 – 240 nM [297] - Tenascin C (TNC), ~ 6 nM [297] - Ubiquitin, 20 pM – 1.6 nM [298] Avacta has a pre-clinical stage biopharmaceutical pipeline of Affimer therapeutic candidates, with a focus on oncology target programmed death ligand 1 (PD-L1) [299]
DARPins (designed ankyrin repeat proteins)	A single ankyrin domain is 3.5 kDa, so DARPins vary from 14-21 kDa depending on the number of repeats they contain	DARPins consist of multiple connected ankyrin molecules	- Epithelial cell adhesion molecule, ~2 nM [300] - VEGF-A, ~25 pM DARPins have shown good safety and efficacy in ongoing clinical trials for the treatment of macular degeneration diseases [301]
Engineered Kunitz domains	~7 kDa	Disulphide crosslinked serine protease inhibitor, typically of human origin e.g. Kunitz domain of TFPI	- Plasma kallikrein, 10 pM Ecallantide (inhibits kallikrein) received FDA approval in 2009 for the treatment of hereditary angioedema [302]
Nanobodies	~15 kDa	A single variable domain derived from a heavy chain antibody, contains a disulphide bond	- Interleukin 6 (IL-6), ~0.2 pM [303] - Lysozyme, ~500 nM [304] Ablynx currently has 8 Nanobodies in clinical development, and caplacizumab (anti-vWF Nanobody) has been approved for the treatment of acquired thrombotic thrombocytopenic purpura (TTP) [305]

Table 1-3 Summary of selected engineered protein scaffolds.

1.6.1 Affimer proteins

Affimers are small proteins consisting of a scaffold constraining two 9 amino acid variable regions [297]. Affimers are based on two scaffold proteins, type I and type II (Figure 1-6). Type II Affimers have a scaffold based on a consensus sequence of plant-derived phytocystatins (cysteine protease inhibitors). The inhibitory sequences within two loops of the protein have been replaced with 9 randomised amino acids in each loop [289]. Type I Affimer proteins have a scaffold based on the human cystatin, Stefin A. Stefin A naturally uses three surfaces to interact, however the type I scaffold is mutated to have two binding loops, similarly to the type II scaffold [306]. The type I Affimers used in this work had 119 residues and were approximately 14 kDa, while the type II Affimers had 109 residues and were around 13 kDa. Affimer proteins have high thermal stability and are expressed easily in bacterial systems. Affimers represent an alternative tool to conventional antibodies that can be applied to a range of research and clinical applications. Affimer proteins carry many of the same advantages as those associated with other small non-antibody protein scaffolds discussed above.

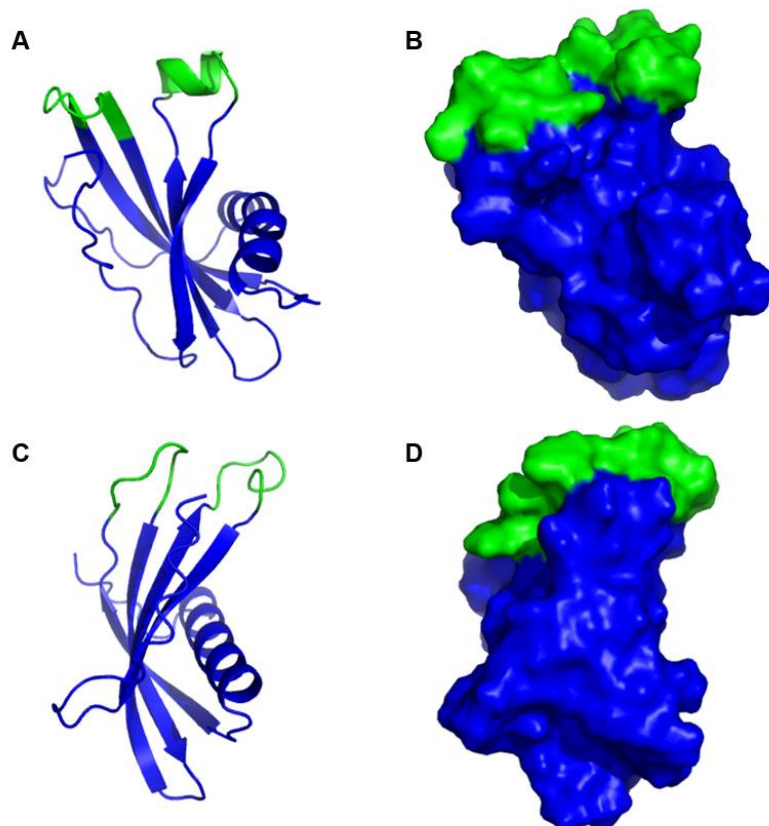


Figure 1-6 Structure of Affimer proteins. The variable region loops are shown in green, and the Affimer scaffold backbone in blue. **A.B.** Type I Affimer scaffold with variable region inserts. **C.D.** Type II Affimer scaffold with variable region inserts.

A disadvantage associated with all small protein scaffolds is a short serum half-life due to renal clearance. Engineered protein scaffolds must therefore bind their target with high affinity to increase local concentration at the target site and resist clearance from the plasma [283]. There are strategies to improve half-life of proteins, such as increasing the protein molecular weight by fusing to an antibody Fc domain, conjugating to albumin or to polyethylene glycol (PEG) [307]. Improvements to protein half-life will be a necessary step in progressing work with Affimers, and is already under investigation by Avacta [308]. However, Affimer proteins that bind to their targets with high affinity are likely to alter half-life to that of the "host" protein.

1.6.2 Current uses of Affimer proteins

The use of Affimer proteins has been investigated in a broad range of applications by others, with protein targets including vascular endothelial growth factor proteins (VEGF), ion channels and human epidermal growth factor receptor 4 (HER-4), among others [297]. As mentioned above, Affimers, like other small engineered protein scaffolds, have the ability to be used similarly to antibodies, and have been used for understanding protein-protein interactions [309, 310] and in a range of sensor technologies [311-314]. Some other examples of uses of Affimer proteins are described below.

Affimer proteins have been used to selectively target members of closely related protein families. Src-Homology 2 (SH2) domains are protein domains that bind specifically to phosphorylated tyrosine residues. There are over 100 SH2-containing proteins encoded by the human genome, and the ability to specifically inactivate each SH2 domain is a barrier in understanding the pathways these proteins are involved in. Tiede et al. (2017) demonstrated the ability of the Affimer phage panning process to isolate high affinity Affimer proteins against specific SH2 containing proteins, with little or no cross reactivity with closely related family members [297].

Tenascin C (TNC) is an extracellular matrix protein that is frequently upregulated in cancer tissues and associated with metastasis. TNC therefore represents a potential marker for tumours in imaging applications and/or therapeutic targeting *in vivo* [315]. Although the large size of antibodies is an advantage in therapeutic

applications, as it confers long blood circulation times, in imaging applications antibodies can be poorly tissue penetrating. Additionally, the long circulation time of antibodies can result in high levels of background signal and poor imaging resolution due to the time taken for non-bound antibody to be cleared from the body [283, 290]. More effective imaging agents are usually small so that they can penetrate tissue and any unbound protein is cleared rapidly from the body, allowing greater resolution in a shorter space of time [283]. Tiede et al. (2017) described a high affinity (K_D 5.7±2.8 nM) TNC-binding Affimer that was C-terminally labelled with fluorescent molecule Rhodamine red for visualisation of TNC in organs from cancer-bearing mice. Anti-TNC Affimers localised to tumours, and showed benefits over TNC antibodies in their more rapid clearance from the mice due to the smaller size of the Affimer proteins [297].

With regards to the clinical application of Affimers, Avacta has a pre-clinical stage pipeline of Affimer therapeutic candidates, with a focus on immune-oncology targets (www.Avacta.com). Affimers have undergone immunogenicity studies (peripheral blood mononuclear cell (PBMC) assays) and shown a low immunogenic response for the type I or type II Affimers tested [316]. As mentioned above, half-life extension studies are ongoing by Avacta.

Affimers, in their potential to bind to any target protein of choice with high affinity and specificity can be used in a wide variety of applications. Affimers, in comparison to antibodies, can be isolated rapidly using only a small quantity of purified target protein, and without the use of animals. The large diversity of Affimer proteins and the library size of $3x10^{10}$ ensures isolation of relevant target-specific Affimers. The easy expression in *E .coli* with a high yield (average 83.3 mg/l) and the high degree of reproducibility between Affimer protein batches make Affimers credible future therapeutic agents [297]. An important aspect of Affimers is the relatively easy modification of these proteins that allows half-life extension or other alterations that may be necessary for clinical use. Taken together, Affimers are promising alternatives to antibodies in a variety of applications, and further work is required to ensure the safety and efficacy of Affimer proteins in clinical applications.

1.7 Hypothesis and aims

Fibrin clot susceptibility to lysis has implications in both bleeding and thrombotic disorders. Formation of an intravascular obstructive thrombus can result in end organ damage, and treatment is aimed at reperfusion to regain blood flow to the affected vessels followed by prophylactic therapy to prevent re-occlusion. Potential therapies aimed at alleviating hypofibrinolysis by targeting the fibrin network have been explored, and this strategy may have particular benefit in subpopulations at increased risk of thrombosis due to hypofibrinolysis.

Conversely, in trauma or surgical associated bleeding, or that resulting from haematological disorders, limiting fibrin clot lysis to stabilise the clot is a key strategy. Anti-fibrinolytic agents have traditionally been used prophylactically before surgery, following traumatic injury, and as adjunctive therapy in individuals with haemophilia. Other fibrin-targeted strategies include fibrin sealants, and novel technologies including a FXIII mimetic have been proposed.

Considering the potential future therapeutic benefits of targeting the fibrin network, I hypothesised that fibrinogen-specific Affimers represent a tool to modulate the fibrinolytic process.

The aim of this work was to investigate the use of Affimer proteins in altering fibrin clot structure/lysis, with the long term view that they may constitute new therapeutic agents for the treatment of thrombotic and/or bleeding disorders.

Objectives:

1. Isolate fibrinogen-specific Affimer proteins by screening the Affimer phage display library against purified fibrinogen
2. Investigate the effects of fibrinogen-specific Affimer proteins on fibrinolysis, using Affimers in purified, plasma, and whole blood systems
3. Characterise the mechanistic pathways for Affimer-mediated modulation of clot lysis

Chapter 2 Experimental design, materials and methods

2.1 Materials

The source and composition of frequently used reagents and buffers:

Material	Source/composition
2TY media	For 1L: 10 g yeast extract; 16 g tryptone; 5 g NaCl
2TY carb	2TY media with the addition of 100 μg/ml carbenicillin
10X casein blocking buffer	Sigma-Aldrich, Dorset, UK
2X casein blocking buffer	10X casein blocking buffer diluted in PBS
Agar	Sigma-Aldrich, Dorset, UK
Acetone	Sigma-Aldrich, Dorset, UK
$CaCl_2$	Sigma-Aldrich, Dorset, UK
Cacodylate acid	Thermo Fisher Scientific, Loughborough, UK
Carbenicillin	Thermo Fisher Scientific, Loughborough, UK
DMSO	Sigma-Aldrich, Dorset, UK
EDTA	Sigma-Aldrich, Dorset, UK
Glu-plasminogen	Enzyme Research Laboratories, Swansea, UK
Glutaraldehyde	Sigma-Aldrich, Dorset, UK
Glycerol	Sigma-Aldrich, Dorset, UK
Glycine	Thermo Fisher Scientific, Loughborough, UK
H_2SO_4	Sigma-Aldrich, Dorset, UK
HCl	Sigma-Aldrich, Dorset, UK
Imidazole	Sigma-Aldrich, Dorset, UK
LB media	Per 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl
LB agar	LB media with the addition of 20 g agar per litre
LB carb	LB media or LB agar with the addition of 100 μg/ml carbenicillin
Methanol	Thermo Fisher Scientific, Loughborough, UK
Multiskan Go plate reader	Thermo Fisher Scientific, Loughborough, UK
Na_2HPO_4	Sigma-Aldrich, Dorset, UK
NaCl	Thermo Fisher Scientific, Loughborough, UK
NaH_2PO_4	Sigma-Aldrich, Dorset, UK
Nanodrop	Nanodrop, Wilmington, USA
Normal pool plasma	First Link UK Ltd., Birmingham, England
Permeation buffer, PB	50mM Tris, 100mM NaCl, pH 7.4
PBS	Tablets, 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4; Sigma-Aldrich, Dorset, UK
PBST	PBS with the addition of 0.1% v/v Tween-20
PEG	Polyethylene glycol, Sigma-Aldrich, Dorset, UK
Plasmin	Sigma-Aldrich, Dorset, UK
Skimmed milk powder	Marvel foods
Sodium azide	Sigma-Aldrich, Dorset, UK
Tetracycline	Sigma-Aldrich, Dorset, UK
Thrombin	Merck Millipore, Watford, UK
tPA	Technoclone, Pathway Diagnostics; Dorking, UK
Tris-base	Thermo Fisher Scientific, Loughborough, UK
Tris-HCl	Thermo Fisher Scientific, Loughborough, UK
Tryptone	Sigma-Aldrich, Dorset, UK
Tween-20	Thermo Fisher Scientific, Loughborough, UK
Yeast extract	Sigma-Aldrich, Dorset, UK

2.2 Phage display to isolate fibrinogen-specific Affimers

As detailed earlier, Affimers are small proteins consisting of a scaffold backbone which constrains two variable 9 amino acid regions. To isolate fibrinogen-specific Affimers, a phage library was screened against purified fibrinogen.

2.2.1 Fibrinogen purification

Plasminogen depleted human fibrinogen (Calbiochem, Merck Millipore, Watford, UK) was purified by affinity chromatography using calcium dependent IF-1 monoclonal antibody (10 mg, Kamiya Biomedical; Seattle, USA) [317] and an ÄKTA Avant 25 protein purification system (GE Healthcare, Little Chalfont, UK) as previously described [24, 153]. Briefly, fibrinogen was dissolved in equilibration buffer (20 mM Tris, 300 mM NaCl, 1 mM $CaCl_2$ in H_2O, pH 7.4) before loading onto a CNBr-activated sepharose 4B column (GE Healthcare, Little Chalfont, UK). Wash buffers were composed of: wash buffer I (20 mM Tris, 1 M Nacl, 1 mM $CaCl_2$ in H_2O, pH 7.4) and wash buffer II (50 mM Na-Acetate, 300 mM NaCl, 1 mM $CaCl_2$, H_2O, pH 6) before applying elution buffer (20 mM Tris, 300 mM NaCl, 5 mM EDTA, H_2O, pH 7.4). All buffers were filtered 0.2 µm and degassed prior to use.

Fibrinogen elution fractions were pooled following affinity chromatography and concentrated using 100 kDa molecular weight cut-off Vivaspin20 concentrator columns (Generon, Berkshire, UK). Fibrinogen sample was spun until the concentration reached approximately 5 mg/ml as measured on a Nanodrop ND-1000 spectrophotometer measuring absorbance at 280 nm and applying mass extinction coefficient 15.1 for fibrinogen. The pooled fibrinogen was then dialysed into permeation buffer (PB) using Pur-A-Lyzer dialysis tubes (Sigma-Aldrich, Dorset, UK). Samples were dialysed overnight at 4°C with a total of three buffer changes.

Purified fibrinogen was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see section 2.9.1) to assess protein integrity, and used in turbidity and lysis assays (see section 2.5.2) to ensure it was functional before use.

2.2.2 Biotinylation of fibrinogen

Fibrinogen was biotinylated using EZ-Link NHS-SS-Biotin (Thermo Fisher Scientific, Loughborough, UK). IF-1 purified fibrinogen was incubated with NHS-SS-Biotin in dimethyl sulfoxide (DMSO) for 1 h at room temperature, according to manufacturer's instructions. Excess non-reacted biotin was removed using Zeba Spin desalting columns (Thermo Fisher Scientific, Loughborough, UK). Biotinylated fibrinogen was mixed with an equal volume of 80% glycerol and stored at -20 °C.

2.2.2.1 Confirmation of fibrinogen biotinylation

An enzyme-linked immunosorbent assay (ELISA) was performed to confirm that the fibrinogen had been successfully biotinylated. 50 µl phosphate-buffered saline (PBS) was added to three wells of a Nunc-Immuno MaxiSorp strip (Thermo Fisher Scientific, Loughborough, UK). 1 µl and 0.5 µl of biotinylated fibrinogen was added to two of the wells and incubated overnight at 4°C, leaving one well with only PBS as a control. Wells were washed three times with 300 µl PBS-Tween (PBST, PBS with 0.1% Tween-20) on a plate washer (TECAN Hydroflex, Tecan Group Ltd, Switzerland) after the overnight incubation. 250 µl 10X casein blocking buffer was added to each well and incubated at 37 °C for 3 h, before washing each well three times with 300 µl PBST on a plate washer. High Sensitivity Streptavidin-HRP (Thermo Fisher Scientific, Loughborough, UK) was diluted 1/1000 in 2X casein blocking buffer and 50 µl added to each well and incubated for 1 h at room temperature on a vibrating platform shaker. Wells were washed six times with 300 µl of PBST on a plate washer and then 50 µl per well of tetramethylbenzidine (TMB) (SeramunBlau, Seramun Diagnostica, Heidesee, Germany) was added to each well, and allowed to develop before measuring absorbance at 620 nm on a plate reader.

2.2.3 Phage panning

Collaborators from the Astbury Centre for Structural Molecular Biology group at the University of Leeds developed a phage display library in a non-antibody binding protein termed Affimer [289]. The Affimer protein scaffold is based on a consensus sequence of plant cysteine protease inhibitor. A phage display library was constructed in this scaffold by insertion of two variable peptide regions, the

library comprises 3 x 10^{10} clones. The following method was used for the isolation of fibrinogen-binding Affimers.

Streptavidin coated wells were blocked with 300 µl of 2X casein blocking buffer (Thermo Fisher Scientific, Loughborough, UK) overnight at 37 °C. The wells were then washed three times with 300 µl PBST, before the addition of 100 µl of 2X casein blocking buffer to all wells. The phage were first put through "pre-panning", to remove any phage that may have bound to the wells of the plate. To pre-pan the phage, 5 µl of phage library was added to one of the four blocked streptavidin coated wells and incubated for 40 min on a microplate shaker. After 40 min, the phage-buffer mix from the first pre-pan well was transferred to the second pre-pan well, and incubated for 40 min on a microplate shaker. This was repeated a third time, before moving the phage-buffer to the final blocked well of the plate, which had been incubated with 10 µl of biotinylated fibrinogen for 2 h at room temperature and then washed six times with 200 µl PBST. The phage library was incubated with the biotinylated fibrinogen for 2 h at room temperature on a microplate shaker.

A fresh culture of ER2738 *Escherichia coli* cells was made by diluting an overnight culture of cells 1/15 into 8 ml of 2TY media containing 12 µg/ml tetracycline and incubating for 1 h at 37 °C with shaking at 230 rpm to an OD_{600nm} of about 0.6. The panning well was then washed six times with 300 µl of PBST on a plate washer to remove non-fibrinogen-binding phage. Fibrinogen-binding phage were eluted by addition of 100 µl of 0.2 M glycine, pH 2.2 to the panning well and incubating for 10 min at room temperature. 15 µl of 1 M Tris-HCl, pH 9.1 was added to the well to neutralise, and then the contents of the well were added to the 8 ml *E. coli* cells. Any phage remaining in the panning well were eluted by the addition of 100 µl of triethylamine (Sigma-Aldrich, Dorset, UK) and incubation for 6 min at room temperature. A total of 50 µl Tris-HCl, pH 7 was then added to the well, mixed, and the contents of the well added to the 8 ml cells to elute any remaining phage. The cells were incubated for 1 h at 37 °C with shaking at 90 rpm. Following incubation, 1 µl of the phage-infected ER2738 cells was plated onto a lysogeny broth (LB)-carb plate (LB agar containing 100 µg/ml carbenicillin) and incubated overnight at 37 °C. The remaining cells were centrifuged at 3000x g for 5 min to resuspend in a smaller volume and plated on LB carb plates for

overnight incubation at 37 °C. The 1 µl plate was used to calculate total number of phage present in the 8 ml of culture, whilst the other plate was scraped by addition of 5 ml 2TY-carb (2TY media containing 100 µg/ml carbenicillin). The scraped bacteria-media mix were added to a 50 ml falcon tube, diluted to OD_{600nm} of ~0.2 and incubated at 37 °C for 1 h with shaking at 230 rpm. M13K07 helper phage (New England Biolabs, Ipswich, US) were added in order to generate a new library for the second round of panning and incubated at 37 °C with shaking at 90 rpm for 30 min. The bacterial culture was centrifuged at 3500x g for 10 min, and the phage-containing supernatant transferred to a fresh tube, ready for the second panning round. Supernatant not needed immediately for the second round of panning was precipitated by addition of 2 ml of PEG-NaCl precipitation solution (20% w/v polyethylene glycol 8000, 2.5 M NaCl) and incubated overnight at 4°C. Centrifugation at 4816x g for 30 min pelleted the phage, which was then resuspended in 320 µl of Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8), centrifuged again at 16,000x g for 10 min. The phage-containing supernatant was diluted with 50% glycerol and stored at -80 °C. In total, four rounds of panning were performed, summarised in Figure 2-1A. After the fourth panning round, phage particles were eluted from the panning well (containing biotinylated fibrinogen) as before, and used to infect a culture of ER2738 cells as before. A range of volumes of culture (0.1 µl, 1 µl, 10 µl and 100 µl) were plated onto LB-carb plates, and the remaining cells centrifuged and plated as described above, prior to phage ELISA.

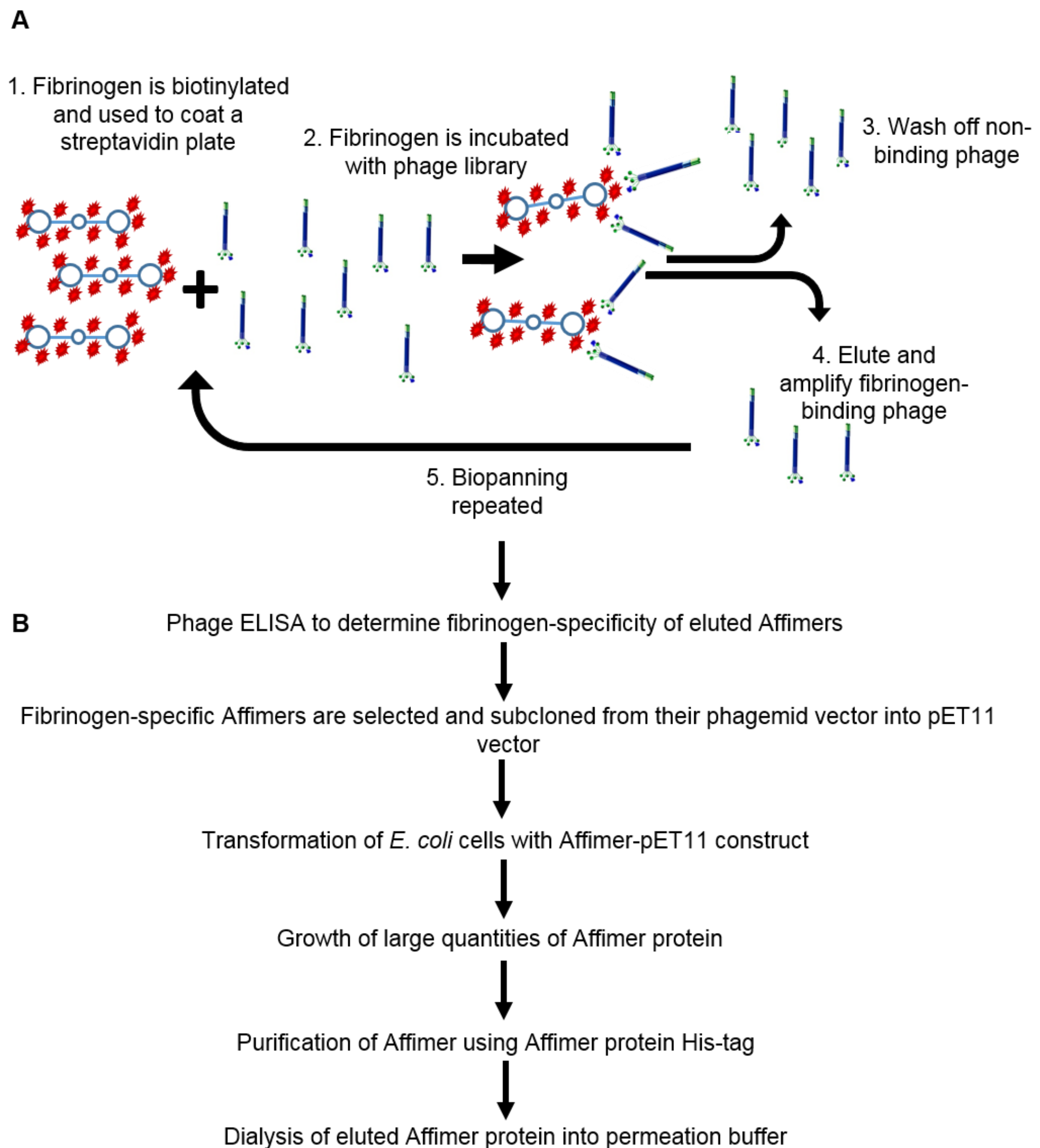


Figure 2-1 Overview of the phage panning method. A. Fibrinogen was first IF-1 purified, and then biotinylated. An ELISA was used to confirm that the protein was biotinylated. Biotinylation enables immobilisation of fibrinogen on a streptavidin-coated plate during the phage panning process. Phage library was then incubated with fibrinogen-coated wells, and non-binding phage washed off. This panning procedure was repeated to enrich for fibrinogen-specific binders. After four rounds of panning, fibrinogen-binding Affimers were eluted. **B.** After fibrinogen-binding Affimers were eluted, their fibrinogen-specificity was determined using a phage ELISA. Fibrinogen-specific Affimers were picked, subcloned from their phagemid vector into pET11, and used to transform *E. coli* cells for protein production. Pure, phage free Affimer protein was purified from *E. coli* cells using the Affimer protein His-tag. Finally, Affimer protein was dialysed into permeation buffer to allow use in downstream applications.

2.2.4 Phage ELISAs to determine fibrinogen specificity of Affimers

2.2.4.1 Preparation of phage

Individual colonies were picked following 4 rounds of panning into a 96-well V-bottom deep well plate containing 200 µl of 2TY-carb, and incubated overnight at 37 °C on a microplate shaker at 1050 rpm. A 10 µl aliquot of each of the cultures was transferred to a new 96 well plate containing 200 µl of 2TY-carb and incubated for 1 h at 37 °C with shaking at 1050 rpm. M13K07 helper phage (titre 10^{14}/ml) (New England Biolabs, Ipswich, US) was diluted 1/1000 in 2TY-carb and 10 µl was added to the freshly grown cultures. The mixture was incubated for 30 min at room temperature with shaking at 450 rpm. Kanamycin (Thermo Fisher Scientific, Loughborough, UK) was added to each well to a final concentration of 50 µg/ml, and the plate incubated overnight at room temperature with shaking at 750 rpm. Phage infected cultures were centrifuged at 3500x g for 10 min, and the phage-containing supernatant was removed directly into the ELISA plate to test for binding to fibrinogen.

2.2.4.2 Phage ELISA

Streptavidin-coated 96 well plates (Thermo Fisher Scientific, Loughborough, UK) were blocked with 200 µl 2X casein blocking buffer overnight at 37 °C. The plates were washed once with 300 µl per well of PBST on a plate washer, prior to incubation with 50 µl biotinylated fibrinogen diluted 1/1000 in 2X casein blocking buffer. The diluted biotinylated fibrinogen was added to the first six columns of the 96 well plate, and blocking buffer added to the last six columns as a negative control. After 1 h incubation at room temperature on a microplate shaker, wells were washed once with 300 µl PBST and then blocked with 10 µl per well of 10X casein blocking buffer. 40 µl of phage-containing supernatant was added to the plate so that each phage was tested against the fibrinogen and the negative control, and incubated for 1 h on a microplate shaker at room temperature. Following another round of washing with 300 µl PBST, phage were detected by addition of 50 µl per well of a 1/1000 dilution of Anti-Fd-Bacteriophage-HRP (Seramun Diagnostica, Heidesee, Germany) in 2X casein blocking buffer, which was incubated for 1 h at room temperature. Wells were washed ten times with 300 µl of PBST on a plate washer, and then 50 µl of TMB was added to the wells and allowed to develop, before reading the plate at 620 nm.

2.2.5 Affimer expression and purification

2.2.5.1 Affimer protein production

To obtain pure, phage-free protein, Affimers of interest were produced by subcloning the coding regions into pET11 vector and expressing the Affimer in *E. coli* BL21 cells as described previously [289, 318].

Following four rounds of phage panning and selection of fibrinogen-specific binders with phage ELISA, overnight cultures of selected binders were grown, and the DNA extracted using QIAprep Spin Miniprep Kit (Qiagen; Sollentuna, Sweden) following the manufacturer's instructions. Plasmid DNA was sent for sequencing by Beckman Coulter to identify unique-binders. The Affimer-coding sequence of unique binders was amplified by polymerase chain reaction (PCR) from the phagemid vector using the following primers and thermocycling conditions:

Forward Primer 5' – ATGGCTAGCGGTAACGAAAACTCCCTG
Reverse Primer for Affimer without a C-terminal cysteine 5' –
TACCCTAGTGGTGATGATGGTGATGC
Reverse Primer for Affimer with a C-terminal cysteine 5' -
TTACTAATGCGGCCGCACAAGCGTCACCAACCGGTTTG

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 s	1
Denaturation Annealing Extension	98 °C 54 °C 72 °C	20 s 20 s 20 s	30
Final Extension Hold	72 °C 4 °C	10 min Hold	1

DpnI (New England Biolabs, Ipswich, US) was then added to each of the PCR reaction tubes to remove *dam* methylated template DNA. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. PCR-amplified Affimer sequences and pET11 plasmid were then restriction digested with NheI and NotI (both New England Biolabs, Ipswich, US) restriction enzymes. Ligation of the NheI-NotI digested Affimer DNA inserts into the pET11 vector was then performed by incubating 75 ng of pET11 DNA with 25 ng of insert DNA with T4 DNA ligase (New England Biolabs, Ipswich, US) overnight at room temperature. XL1-Blue supercompetent cells (Agilent Technologies; Stockport, UK) were then transformed with this Affimer/pET11 construct. 1 μl of ligation mix was incubated with 10 μl of competent cells on ice for 30 min. Samples were then heat shocked by placing in a water-bath at 42°C for 45 s, before incubating on ice for 2 min. 180 μl of pre-heated SOC medium (Thermo Fisher Scientific, Loughborough, UK) was then added and incubated at 37 °C for 1 h with shaking at 250 rpm. 100 μl of each transformation mixture was then plated onto LB-carb plates and incubated overnight at 37 °C. Single colonies were picked into 5 ml of LB-carb media (LB media containing 100 μg/ml carbenicillin) and grown overnight at 37 °C with shaking at 230 rpm, and the plasmid DNA extracted using QIAprep Spin Miniprep Kit (Qiagen; Sollentuna, Sweden) according to the manufacturer's instructions. The purified plasmid DNA was sent for sequencing to confirm correct Affimer DNA sequence in the pET11 vector.

To produce large quantities of the Affimer protein, BL21 Star chemically competent *E.coli* cells (Life Technologies, Paisley, UK) were transformed with the Affimer-pET11 construct using the transformation procedure described above. Colonies of transformants were picked into 2 ml LB-carb with 1% glucose and grown overnight at 37 °C with shaking at 230 rpm. Overnight culture was used to inoculate 50 ml of LB-carb. The cultures were incubated at 37 °C with shaking at 230 rpm until they reached an OD_{600nm} of ~0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich, Dorset, UK) was added to the cultures to a final concentration of 0.1 mM which were then incubated for a further 6 h at 25 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 4816x g for 15 min, and the supernatant discarded. Cell pellets were resuspended in 1 ml total volume of lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl;

20 mM imidazole; 10% glycerol; pH 7.4) supplemented with Lysozyme (Thermo Fisher Scientific, Loughborough, UK), Triton X-100 (Sigma-Aldrich, Dorset, UK), Benzonase Nuclease (Merck Millipore, Watford, UK) and EDTA-free Halt Protease Inhibitor (Thermo Fisher Scientific, Loughborough, UK). Resuspended cell pellets were then transferred to nickel-nitrilotriacetic acid (Ni-NTA) resin (Amintra, Expedeon Ltd, Cambridge, UK) and incubated for 2 h at room temperature on a carousel mixer. Samples were centrifuged at 1000x g for 1 min and the supernatant removed, before the resin was washed with wash buffer (50 mM NaH_2PO_4; 500 mM NaCl; 20 mM Imidazole; pH 7.4) to remove unbound proteins. Resin was washed until Nanodrop readings at 280 nm consistently read <0.09. Affimers were eluted by resuspending the resin in 500 µl elution buffer (50 mM NaH_2PO_4, 500 mM NaCl; 300 mM Imidazole; 10% Glycerol; pH 7.4) and incubating for 5 min. Resin was centrifuged at 1000x g for 1 min to sediment the resin, and eluate containing Affimer protein was collected. Affimer protein was dialysed into PB overnight at 4°C in Pur-A-Lyzer dialysis tubes (Sigma-Aldrich, Dorset, UK), with two buffer changes during this time. The process of phage panning and subsequent subcloning, expression and purification of Affimer protein is summarised in Figure 2-1B.

2.2.5.2 Size exclusion chromatography

Size exclusion chromatography (SEC) was performed with the assistance of Dr Iain Manfield at the Centre for Biomolecular Interactions, University of Leeds. SEC was used as a further purification step following protein purification with Ni-NTA slurry as described above.

SEC was performed using an ÄKTA explorer protein purification system (GE Healthcare Life Sciences) with a Superdex 200 (16/600) column (GE Healthcare Life Sciences). After equilibration of the column and loading of the sample, absorbance was monitored at 220, 260 and 280 nm throughout. Selected elution fractions were chosen from the peaks generated during the size exclusion protocol and analysed by SDS-PAGE (as described in 2.9.1). Fractions containing only a single band corresponding to the molecular weight of the Affimer were pooled and dialysed into PB overnight at 4°C in Pur-A-Lyzer dialysis tubes (Sigma-Aldrich, Dorset, UK), with two buffer changes during this time.

2.2.5.3 Isolation of fibrinogen-specific Affimers by Avacta

Collaborators at Avacta Life Sciences Ltd. (Wetherby, UK) performed a screen for fibrinogen-binding Affimers using a phage library in a human version of the Affimer scaffold. These type I fibrinogen-binding Affimers were isolated and subcloned by Avacta. Type I Affimers are based on a naturally occurring human protease inhibitor Stefin A [306]. Affimers with this scaffold were used on the basis that they will be potentially be less immunogenic if used in future human studies.

2.3 Affimer-fibrinogen ELISA

A 96 well Nunc-Immuno Maxisorp ELISA plate (Thermo Fisher Scientific, Loughborough, UK) was coated overnight with 100 µl of 1 µg/ml Affimer protein in coating buffer (2.5 mM $NaH_2PO_4.2H_2O$, 7.5 mM $Na_2HPO_4.2H_2O$, 145 mM NaCl, pH 7.2). Controls included i) a non-fibrinogen-binding Affimer (anti-SUMO), ii) scaffold-only protein, iii) wells containing coating buffer only. After overnight incubation, wells were washed three times with 250 µl wash buffer (WB, coating buffer with 2% v/v Tween-20 and 350 mM NaCl), and then blocked with 200 µl of 1% bovine serum albumin (BSA) in WB for 60 min at 37 °C. After blocking, 100 µl of 1 µg/ml IF-1 purified fibrinogen diluted in WB was added to the wells and incubated for 2 h at room temperature. Wells were washed three times with 250 µl WB, prior to addition of a goat polyclonal anti-fibrinogen-HRP antibody (Abcam, Cambridge, MA) diluted 1/16,000 in WB and incubation at room temperature for 1 h on a plate shaker. The plate was washed three times with 250 µl WB before developing with the addition of o-phenylenediamine dihydrochloride (OPD) reagent tablets (Alere Inc., Massachusetts, US), made up with H_2O and H_2O_2. 100 µl of OPD solution was added to wells, and the reaction stopped with the addition of 100 µl 1.5 M H_2SO_4. Absorbance was measured at 490 nm on an ELx-808 microplate reader (BIO-TEK Instruments Inc., Winooski, US).

2.4 Clauss method for determining plasma fibrinogen concentration

Fibrinogen levels in normal pool plasma were determined by the Clauss method using a Start 4 haemostasis analyser (Diagnostica Stago; Theale, UK) and the fibri-prest automate kit (Diagnostica Stago; Theale, UK) according to the manufacturer’s instructions. The fibrinogen concentration of a plasma sample is

determined in this method by measuring the time taken for a clot to form under the influence of excess thrombin. Under these conditions the fibrinogen concentration is rate limiting, so the clotting time can be used as a measure of the amount of fibrinogen in the sample.

Control plasmas N and P were reconstituted with 1 ml dH_2O and left for 30 min at room temperature. 2 ml dH_2O were added to Fibri-Prest Automate reagent, which contains thrombin, and also left for 30 min. Control plasmas and Fibri-Prest were homogenised prior to sample measurement. A standard curve of known fibrinogen concentration was prepared by diluting Unicalibrator in STA-Owren-Koller buffer as per the manufacturer's instructions. Plasma sample ([fibrinogen] to be determined) and control plasmas N and P were diluted 1/20 in STA-Owren Koller buffer.

To perform the assay, cuvette strips (Diagnostica Stago; Theale, UK) were pre-warmed to 37 °C, and a steel ball bearing (Diagnostica Stago; Theale, UK) placed in each well. 100 µl of each standard/control plasma/plasma sample was added to the wells of the cuvette strip in duplicate. The cuvette strips were then placed into the Start 4 haemostasis analyser which oscillates the steel ball bearings from side to side. 50 µl of fibri-prest automate solution was added to each well of the cuvette strip to initiate clot formation. As the clot forms, the movement of the steel ball is impeded. The fibrinogen concentration of the plasma sample was determined by comparing the time taken for the clot to form against the calibration curve of known fibrinogen concentrations.

2.5 Turbidimetric assays, in plasma and purified systems

The effect of the Affimers on clot formation and lysis was determined by turbidimetric analysis, in both plasma and purified systems. Turbidity experiments were used to assess the effect of Affimers on clot formation (lag time) and clot turbidity (maximum OD). Turbidity and lysis assays were used to assess the effect of Affimers on the lysis of clots (lysis time).

Turbidimetric assays in a plasma and purified system were first optimised to investigate the effect of varying concentrations of thrombin, tPA and plasminogen. These optimisation experiments are detailed further in chapter 3.

2.5.1 Optimisation of turbidimetric experiments

2.5.1.1 Plasma

Turbidity assays

Plasma turbidimetric assays were performed by adding 25 µl plasma to a 96 well clear-bottom plate (Greiner Bio One International GmbH) and adding PB to 120 µl. Clotting was initiated by adding 30 µl of activation mix containing 0, 0.03, 0.12, 0.48 or 1.92 U/ml thrombin and 0, 5, 7.5, 10 or 22.5 mM $CaCl_2$ (all concentrations final). Measurements of the optical density at 340 nm were taken at 12 s intervals on a Multiskan Go plate reader at 37 °C.

Turbidity and lysis assays

Plasma turbidimetric assays were performed by adding 25 µl plasma to a 96 well clear-bottom plate and adding PB to 90 µl. Lysis mix containing 83 ng/ml (1.2 nM) tPA was added to each well in a volume of 30 µl, followed by 30 µl of activation mix containing 7.5 or 22.5 mM $CaCl_2$ and 0, 0.03, 0.12, 0.48 or 1.92 U/ml thrombin (all concentrations final). Measurements of the optical density at 340 nm were taken at 12 s intervals on a Multiskan Go plate reader at 37 °C.

2.5.1.2 Purified

For turbidimetric experiments in a purified system, 0.5 mg/ml (1.47 µM) fibrinogen (Calbiochem, Merck Millipore, Watford, UK) was added to the wells of a 96 well clear-bottom plate (Greiner Bio One International GmbH) in a total volume of 90 µl PB. Lysis mix containing 0, 1.5, 3.125, 6.25, 12.5 or 25 µg/ml (0, 16.3, 34.0, 68.0, 135.9, 271.7 nM) plasminogen and 0, 19.5, 39, 78, 156 or 312 ng/ml (0, 0.28, 0.56, 1.11, 2.23, 4.46 nM) tPA was added to each well in a volume of 30 µl, followed by 30 µl of activation mix containing 0.05 U/ml thrombin and 2.5 mM $CaCl_2$ (all concentrations final). Measurements of the optical density at 340 nm were taken at 12 s intervals on a Multiskan Go plate reader at 37 °C.

2.5.2 Optimised plasma turbidimetric assay

Final concentrations chosen for optimised turbidimetric assays were 83 ng/ml (1.2 nM) tPA, 0.03 U/ml thrombin and 7.5 mM $CaCl_2$.

Human plasma samples

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

Affimer proteins were also tested in plasma deficient in FVIII (Haematologic Technologies Inc., Essex, UK), representing high risk bleeding groups. These experiments were performed similarly to those using normal pool plasma, with the exception that in these assays, a mixture of tissue factor and phospholipids at a final concentration of 5 pM (PPP-Reagent HIGH, Thrombinoscope, Maastricht, The Netherlands) was used to initiate thrombin generation.

Urokinase type tissue plasminogen activator

To test the fibrin specificity of Affimer F5-induced prolongation on clot lysis, turbidimetric experiments were performed with urokinase in place of tPA, since urokinase induced plasminogen-plasmin conversion is not fibrin dependent. These assays were performed as described above in plasma experiments with 1.06 µg/ml (20 nM) urokinase (Technoclone, Pathway Diagnostics; Dorking, UK) instead of tPA.

Mouse plasma

Turbidimetric experiments were also performed with mouse plasma (Seralab, Bio-IVT). Affimer protein was incubated with 25 µl mouse plasma in PB for 15 min at room temperature, prior to the addition of tPA (225 ng/ml, 3.2 nM) for a further 20 min incubation, and initiation of clotting with thrombin (0.03 U/ml) and $CaCl_2$ (7.5 mM) (all concentrations final). Measurements of the optical density at 340 nm were taken at 12 s intervals on a Multiskan Go plate reader at 37 °C.

Turbidimetric assays with Affimer variable region peptides

Four linear peptides of the same sequence as the variable regions of Affimer proteins F5 (chapter 4) and B3 (chapter 5) were synthesized by Thermo Fisher Scientific with peptide purity >95%. These peptides were reconstituted in PB and tested for an effect on clot formation and lysis using the plasma turbidimetric protocol.

2.5.2.1 Plasma assays with lysis mix added to pre-formed clots

Plasma turbidimetric experiments were also performed in which lysis mix was added to the top of fully formed clots. Performing the experiment in this way allows clot formation to occur without concurrent lysis.

In these experiments, 25 µl of plasma was added to the wells of a 96-well plate, and incubated for 30 min at room temperature with PB in a total volume of 60 µl. After 30 min, 30 µl of activation mix containing 0.03 U/ml thrombin and 7.5 mM $CaCl_2$ was added to the wells, and the plate was read every 12 s at 37 °C for 1 h. After 1 h, 30 µl of Affimer was added to the wells of the plate, immediately prior to the addition of 30 µl of a lysis mix containing 83 ng/ml (1.2 nM) tPA (all concentrations final). The plate was read every 12 s at 37 °C.

This experiment allowed assessment of the Affimer's effect on the lysis of fully formed clots formed in the absence of Affimer. A variation of this experiment was also performed in which Affimer was added at the start of the experiment and incubated with plasma, prior to addition of the activation mix. Again, lysis mix was added after 1 h once clot formation had been allowed to proceed.

2.5.3 Purified turbidimetric assay, final (optimised) protocol

Final concentrations chosen for these assays were 3 µg/ml (34 nM) plasminogen, 39 ng/ml (0.56 nM) tPA, 0.05 U/ml thrombin and 2.5 mM $CaCl_2$.

2.5.3.1 Purified experiments with plasminogen/tPA vs plasmin

In order to clarify the mechanism of action of Affimer F5, turbidity and lysis experiments using purified proteins were performed with lysis mix containing either plasminogen and tPA to initiate lysis, or plasmin. Fibrinogen (Calbiochem,

Merck Millipore, Watford, UK) was added to the wells of a 96 well plate (Greiner Bio One International GmbH) at 0.5 mg/ml (1.47 μM) with Affimer protein, in a total volume of 90 μl PB. After 30 min incubation at room temperature, 30 μl of activation mix was added containing 0.05 U/ml thrombin and 2.5 mM calcium. Measurements of the optical density at 340 nm were taken at 12 s intervals on a Multiskan Go plate reader at 37 °C for 1 h. After 1 h of clotting, 30 μl lysis mix was added to the wells which contained either 3 μg/ml (34 nM) plasminogen with 39 ng/ml (0.56 nM) tPA, or 3 μg/ml (36 nM) plasmin. Measurements were then taken again every 12 s at 37 °C to follow clot lysis.

2.5.4 Turbidimetric parameters

Several clot parameters can be calculated from the turbidimetric assay curves generated [319] (Figure 2-2).

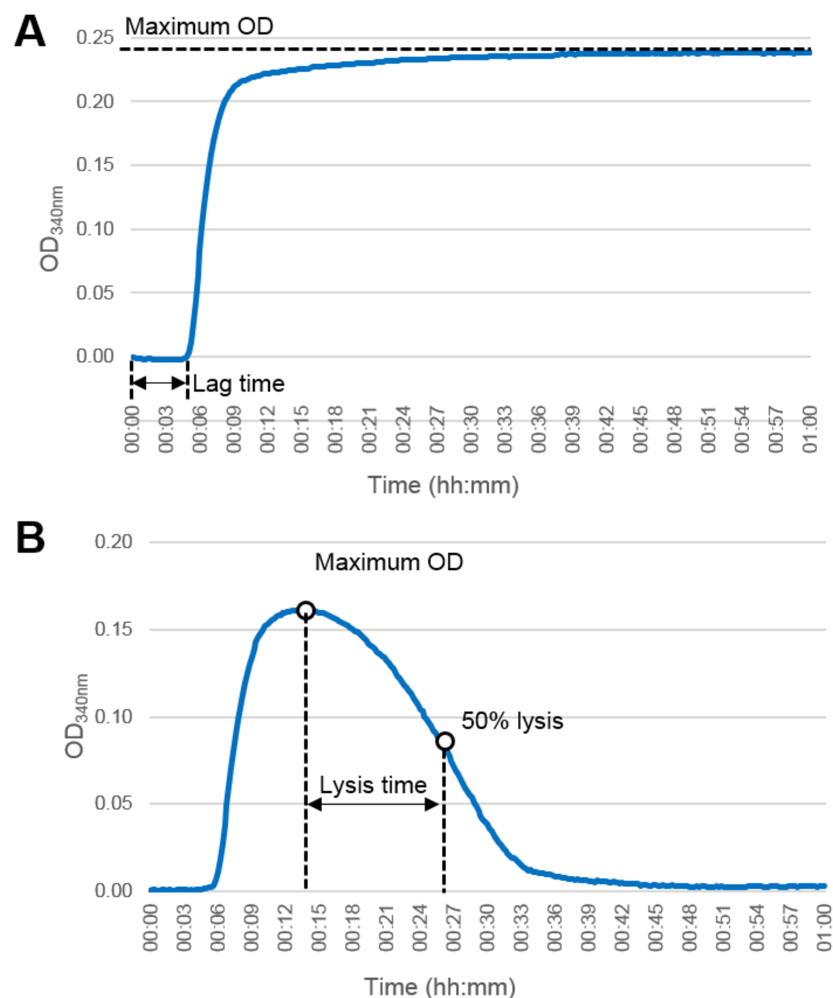


Figure 2-2 Turbidimetric assay clotting and lysis parameters. A. Example of a curve generated from a turbidity experiment. The maximum OD and lag time are parameters calculated from turbidity experiments. **B.** An example of a curve generated from a turbidity and lysis experiment, and the determination of lysis time from the curve.

The following parameters were calculated using an in-house generated excel spreadsheet. The lag time corresponds to the initial stages of clot formation, and is the time taken for the fibrinogen to convert to fibrin monomers and lateral aggregation of protofibrils into fibrin fibres. In all experiments, the lag time was calculated as the time taken for the OD_{340nm} to exceed 0.01 (Figure 2-2A). Maximum absorbance was taken as the highest absorbance reading recorded after normalising the turbidimetric readings by subtraction of the baseline OD (Figure 2-2A). Maximum OD reflects the thickness of fibrin fibres and also clot density, with a higher maximum OD indicative of thicker fibres or a more dense clot structure. Lysis time was calculated as the time from maximum absorbance to the point at which 50% of the clot had lysed (Figure 2-2B).

2.6 Laser scanning confocal microscopy

The effect of the Affimers on clot structure was determined using laser scanning confocal microscopy (LSCM), in both plasma and purified systems.

2.6.1 Labelling of Affimer proteins for LSCM

Affimers were fluorescently labelled using Alexa Fluor-488 Microscale Protein Labelling Kit (Invitrogen, Paisley, UK). Affimer proteins were first buffer exchanged into PBS using Zeba Spin de-salting columns (Thermo Fisher Scientific, Loughborough, UK). Affimer protein samples were diluted to 1 mg/ml, and 100 µl of sample added to 10 µl of 1 M sodium bicarbonate (provided with kit) in a reaction tube. Reactive dye (provided with the kit) was reconstituted with water, and the reactive dye solution added to the prepared Affimer sample, mixed, and incubated for 15 min at room temperature covered in foil to protect from light. After incubation, the labelled Affimer was removed from unreacted dye using gel resin and a spin filter provided with the kit. Gel resin was first prepared by re-suspending in PB after removal of the resin storage buffer. This step ensured that the labelled Affimers were eluted in PB and not PBS, for downstream applications. The washed and buffer-exchanged resin was re-suspended by gentle mixing, and 800 µl added to a spin filter which was centrifuged for 15 s at 16000x g to pack the resin. 50 µl of Affimer protein was then added to the prepared spin filter and centrifuged for 1 min at 16000x g. Unreacted dye remained in the gel resin, and the labelled Affimer collected for use in confocal microscopy.

2.6.2 Plasma experiments

A total of 7.5 µl pooled human plasma was incubated with 0.03 mg/ml Alexa Fluor-488 labelled fibrinogen (Invitrogen, Paisley, UK) and Affimer protein for 30 min in a total volume of 30 µl at room temperature. After 30 min, an activation mix containing 5 mM $CaCl_2$ and 0.05 U/ml thrombin in 5 µl was added to each sample, and mixed three times. The mixture (30 µl) was loaded onto an Ibidi 6 channel slide (Thistle Scientific, UK). Samples were prepared in duplicate. Samples were stored overnight in a humidity chamber in the dark to ensure full clot formation. Fibrin networks were visualised on a LSM880 microscope (Carl Zeiss, Welwyn Gardens City, Hertfordshire, UK) using a 40x 1.4 oil objective lens. Z stacks of clots were imaged -30 slices at 0.7 µm intervals (clot thickness 20.3 µm). Fibre count was determined using an in-house generated macro for ImageJ (National Institutes of Health, Bethesda, US). This macro calculates the number of fibres crossing 10 horizontal and 10 vertical lines drawn on top of a confocal image of a clot. From this the average number of fibres/100 µm can be calculated.

2.6.3 Plasma experiments with labelled Affimers

For experiments with labelled Affimers, clots were prepared with plasma as described above, with Alexa Fluor-594 labelled fibrinogen (Invitrogen, Paisley, UK) in place of Alexa Fluor-488 fibrinogen. Also, fluorescently labelled Affimers were used at 5:1 Affimer:fibrinogen molar ratio in place of non-labelled Affimers (fluorescent labelling of Affimers described in 2.6.1). To determine the degree of Affimer incorporation into fibrin networks, yellow pixels were quantified using Colour Pixel Counter Plugin for ImageJ (National Institutes of Health, Bethesda, US). Yellow pixels occur when signal from fibrinogen (red) and Affimer (green) overlay.

2.6.4 Purified experiments

Affimer protein was incubated for 30 min at room temperature with 1 mg/ml IF-1 purified fibrinogen and 0.03 mg/ml Alexa Fluor-488 labelled fibrinogen (Invitrogen, Paisley, UK) in a total volume of 30 µl. After incubation, 5 µl of an activation mix containing 5 mM $CaCl_2$ and 0.05 U/ml thrombin was added to each sample, and mixed three times. 30 µl of the mixture was loaded onto an Ibidi 6 channel slide (Thistle Scientific, UK). Samples were prepared in duplicate and visualised as with the plasma samples.

2.7 Scanning electron microscopy

Clots prepared for scanning electron microscopy (SEM) were made in the lids of 0.6 ml Eppendorf tubes that had been punctured with a needle and the bottom of the lids covered in Parafilm (Sigma-Aldrich, Dorset, UK). Plasma clots were prepared by diluting 50 µl normal pool plasma in 100 µl total volume of PB, with and without Affimer protein. Plasma, PB and Affimer protein were incubated for 30 min at room temperature, before 45 µl of the mix was pipetted into two tubes and 5 µl of activation mix added, containing 2.5 mM $CaCl_2$ and 0.5 U/ml thrombin. After mixing, the 50 µl of plasma/Affimer/activation mix was added to the lid of a prepared Eppendorf tube and allowed to clot for 2 h at room temperature in a humidity chamber. After 2 h the Parafilm was removed from the bottom of the Eppendorf lid, and the lid placed in a beaker containing cacodylate buffer (67 mM cacodylate acid, 2.7 mM HCl, pH 7.4) for 40 min with mixing. Cacodylate buffer was exchanged for fresh buffer twice, each time the clots were washed in the buffer for 40 min with mixing. Clots were then placed in 2% glutaraldehyde in cacodylate buffer for 2 h in a fume hood with mixing, before three 20 min washes in cacodylate buffer. Clots were dehydrated, by placing in increasing concentrations of acetone (30% for 15 min, 50% for 15 min and 70% overnight). The following day, the clots were placed in 80%, 90% and then 95% acetone, each for 15 min, before three 15 min incubations in 100% acetone. Clots were then critical point dried by Martin Fuller in the Faculty of Biological Sciences, University of Leeds using an E3000 critical point dryer (Quorum Technologies Ltd, UK) before mounting onto stubs. Clots were sputter-coated with 5 nm iridium using a Cressington 208 HR (Cressington Scientific Instruments, Watford, UK). Each clot was imaged in five areas, at different magnifications (5000, 10000, 30000, and 50000 x) using a Hitachi SU8230 high performance cold field emission (CFE) SEM (Chiyoda, Tokyo, Japan). Mean fibre thickness was determined by measuring 15 fibres in each image taken at 10K magnification for a particular sample (total of 75 fibres for each sample duplicate) using ImageJ software (National Institutes of Health, Bethesda, US).

2.8 Clot permeation

These experiments were performed by Dr Ramsah Cheah to study the permeation properties of fibrin clots formed in the presence of Affimers.

Clots were prepared by first incubating 50 µl normal pool plasma with 50 µl Affimer protein in PB for 30 min at room temperature. After incubation, 10 µl of an activation mix containing 1 U/ml thrombin and 10 mM $CaCl_2$ was added and mixed briefly with the Affimer/plasma, prior to pipetting 100 µl of the mixture into a “clotting tip”. Clotting tips were prepared by cutting a 4.5 cm length from the tip of a 1 ml Costar burette tip (Sigma-Aldrich, Dorset, UK) and using a scalpel to create a rough interior surface in the tip. Samples were left in clotting tips in a humidity chamber for 2 h at room temperature to allow fibrin clot formation. After 2 h, the clotting tip was connected through a silicon tube to a syringe containing PB with a 4 cm pressure drop. Clots were left for 90 min to allow permeation and washing of the clot with PB (Figure 2-3). To enable calculation of the Darcy constant, drops of permeation buffer were collected into pre-weighed 2 ml tubes which were weighed again every 30 min for 2 h to determine how much buffer had flowed through the clot in every 30 min period. Clot permeability was determined by calculating the Darcy constant (Ks), which is a measure of the average pore size of the fibrin network [320].

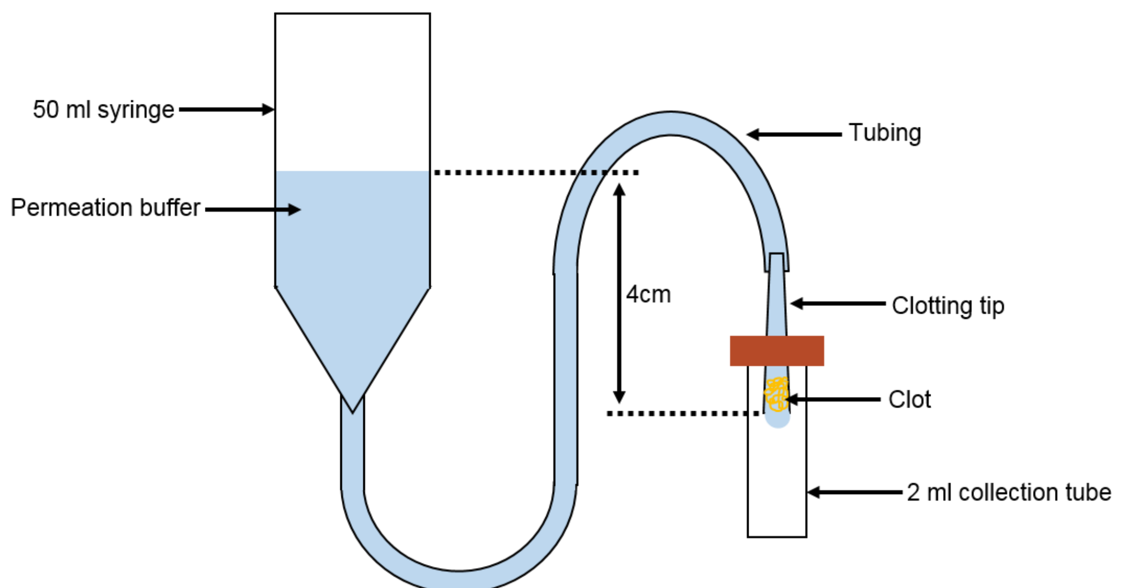


Figure 2-3 Permeation experimental set up.

2.9 SDS-PAGE and Western blotting

2.9.1 SDS-PAGE gels

For SDS-PAGE under reducing conditions, the following standard protocol was used. Samples were run on a 4-12% Bis-Tris gel (Invitrogen, Thermo Fisher Scientific, Loughborough, UK) for resolving smaller proteins (e.g. Affimers) or a 10% Bis-Tris gel (Invitrogen, Thermo Fisher Scientific, Loughborough, UK) for larger proteins (e.g. fibrinogen). Samples were reduced using NuPAGE sample reducing agent and also received NuPAGE lithium dodecyl sulphate (LDS) sample buffer (both Invitrogen, Thermo Fisher Scientific, Loughborough, UK), prior to heating at 95 °C for 10 min. Samples were loaded onto gels in a XCell SureLock electrophoresis system (Invitrogen, Thermo Fisher Scientific, Loughborough, UK). NuPAGE 2-(N-morpholino)ethanesulfonic acid (MES) buffer was used for resolving small molecular weight proteins, and NuPAGE 3-(N-morpholino)propanesulfonic acid (MOPS) for mid-size proteins (both Invitrogen, Thermo Fisher Scientific, Loughborough, UK), each diluted 1/20 in dH_2O prior to use. Gels were typically run for 45 min at 200 V (MES buffer) or 50 min at 200 V (MOPS buffer), or until good separation of the protein standard ladder (Precision Plus protein standard, Bio-Rad Laboratories Ltd., Watford, UK). After running, gels were washed three times for 10 min each in ddH_2O, prior to staining for 1 h with GelCode Blue Safe Protein Stain (Thermo Fisher Scientific, Loughborough, UK). Gels were de-stained overnight and then imaged on a G:Box Chemi XT4 imaging system (Syngene, Cambridge, UK).

2.9.2 Western Blotting

After running as described above, gels were washed for 20 min in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol v/v, pH 8.3) with shaking. Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Loughborough, UK) was prepared by placing in methanol for 15 s, followed by 2 min in ddH_2O, and finally placing in transfer buffer until needed. To assemble a blot, a foam pad soaked in transfer buffer was placed on the black side of a gel holder cassette, and on top of this a filter paper (also soaked in transfer buffer), followed by the gel, the prepared membrane, a filter paper, and a final foam pad (all Bio-Rad Laboratories Ltd., Watford, UK). During assembly of the blotting cassette, all components were kept wet with transfer buffer and bubbles rolled out between each surface with a roller. The assembled cassettes were then

placed in the Mini Trans-Blot western tank (Bio-Rad Laboratories Ltd., Watford, UK). Western blots were run for 1 h at 100 V, with an ice block placed in the tank and a magnetic stirrer.

After transferring, membranes were washed for 10 min in Tris-buffered saline with Tween-20 (TBST, 50 mM Tris, 150 mM NaCl, 0.1% Tween-20 v/v) with shaking, and then incubated with blocking buffer (5% w/v milk powder in TBST) with shaking. Membranes were washed four times for 15 min each with TBST at room temperature with shaking. The membranes were incubated at room temperature with primary antibody diluted in blocking buffer, before washing, as before. Membranes were incubated with secondary antibody in blocking buffer at room temperature with shaking, with the addition of StrepTactin-HRP (Bio-Rad Laboratories Ltd., Watford, UK) diluted 1/5000 for detection of the molecular weight marker. Membranes were washed again, as before, prior to development and visualisation of the blot. Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Loughborough, UK) was used for developing the blots, which were imaged immediately using a G:Box Chemi XT4 imaging system (Syngene, Cambridge, UK). Exact experimental details for fibrinogen and Affimer Western blots are summarised in Table 2-1.

Blotting against:	**Protein loaded**	**Blocking step**	**Primary antibody, incubation time**	**Secondary antibody, incubation time**
Fibrinogen	2 µg	Overnight, 4 °C	Rabbit anti-human fibrinogen (Dako), diluted 1/5000, 2 h	Goat anti-rabbit-HRP (Dako), diluted 1/5000, 1 h
Affimer	2.5 µg	1 h, room temperature	Mouse anti-poly histidine (Roche), diluted 1/200, 1 h	Rabbit anti-mouse-HRP (Dako), diluted 1/1000

Table 2-1 Western blotting details

2.10 Rotational thromboelastometry (ROTEM)

Samples of free-flowing blood were collected from the antecubital vein of healthy volunteers after informed written consent in accordance with the declaration of Helsinki. Ethical approval was obtained from the University of Leeds Medical School Ethical Committee. Blood was collected in 0.109 M sodium citrate, and was used after 30 min rest at room temperature [321]. Measurements were

performed on a ROTEM delta and raw data extracted using Export Tool ROTEM delta V1.3 (ROTEM, Tem International GmBH).

In ROTEM experiments, the blood sample and ROTEM reagents are placed into a cuvette into which a pin is inserted. A 1 mm gap between the pin and the bottom of the cup is bridged by blood. The pin is rotated left and right, and moves freely before the blood has clotted. As the blood clot forms, the movement of the pin is impeded, thus, the rotation of the pin is inversely proportional to clot firmness. A ROTEM trace is produced over time (Figure 2-4), and various clot parameters calculated (described below).

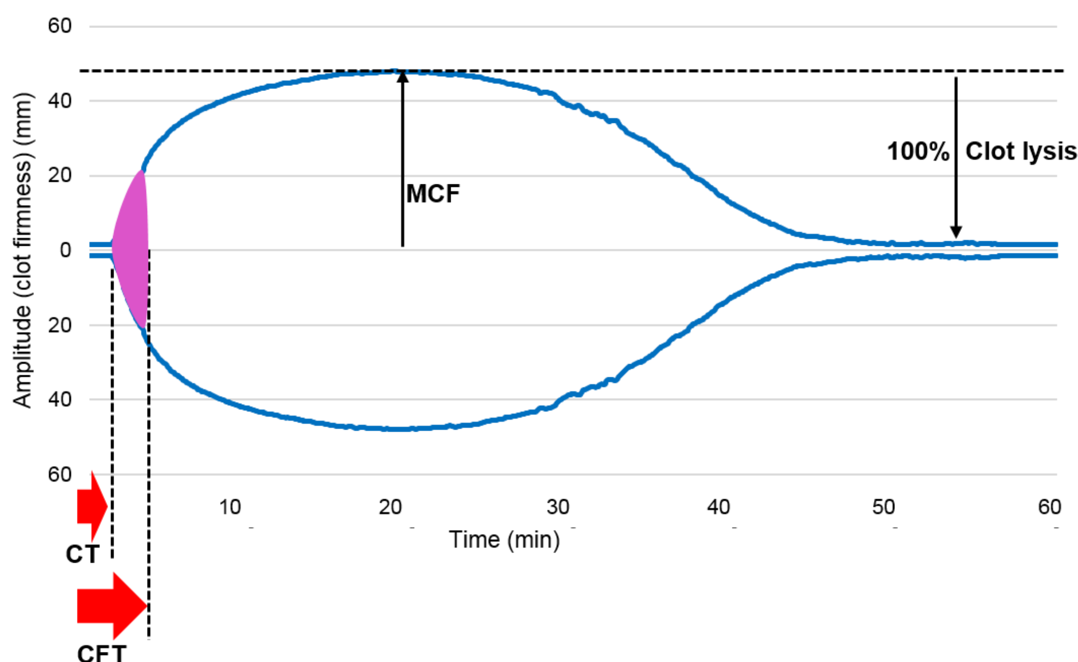


Figure 2-4 ROTEM trace. An example of a reaction curve generated during a ROTEM experiment. The parameters of interest that can be derived from the reaction curve are clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF) and % clot lysis.

2.10.1 EXTEM tests

In EXTEM experiments, coagulation is activated with tissue factor. Samples were re-calcified using 20 µl star-tem reagent, and clotting initiated using 20 µl ex-tem reagent (both ROTEM, Tem International GmBH), which were added to pre-warmed plastic cups (Cup and Pin pro measurement cells, ROTEM, Tem International GmBH). Affimer protein was added to the cup in a total volume of 90 µl in saline solution (0.9% NaCl). EXTEM tests were performed with and

without the addition of 175 ng/ml (2.5 nM) tPA, which was added to the plastic cup before the addition of blood. Finally, 210 µl whole blood was added, mixed once with the pipette, and then the experiment initiated. EXTEM tests were run for up to 5 h at 37 °C.

2.10.1.1 Optimising [tPA] in EXTEM tests

To find an appropriate concentration of tPA to include in EXTEM assays (to assess the effect of Affimers on whole blood lysis), a concentration curve of tPA was used (Figure 2-5). Experiments were performed as described above with tPA added to the reaction mixture to either 70, 140, 350, 700 ng/ml (1 nM, 2 nM, 5 nM or 10 nM). Based on the ROTEM curves generated during this experiment (Figure 2-5), 175 ng/ml (2.5 nM) tPA concentration was chosen, to allow full lysis of clots within a 1 h period.

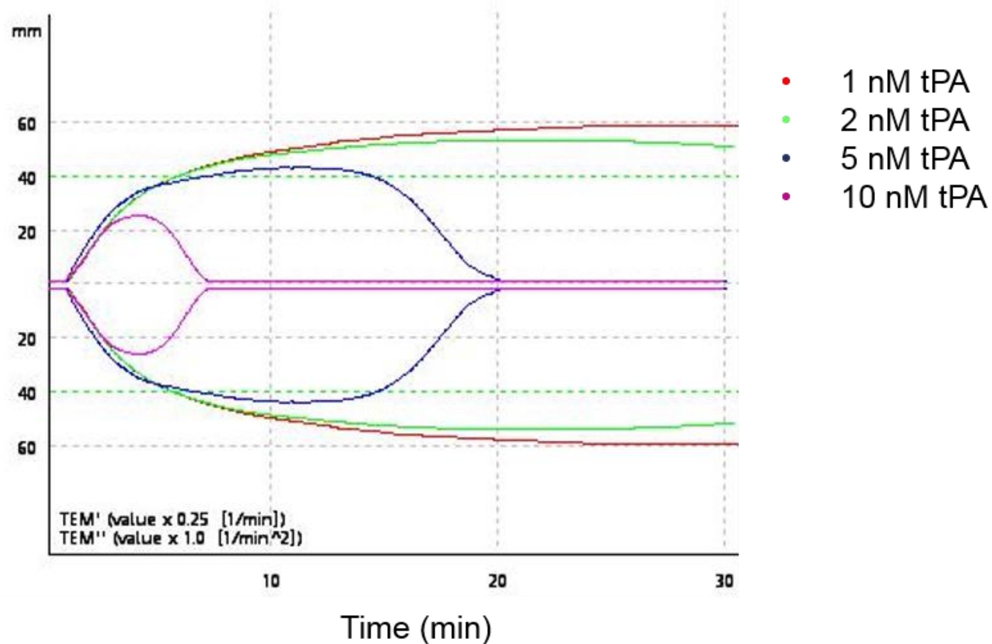


Figure 2-5 tPA concentration curve. Increasing concentrations of tPA were used in ROTEM EXTEM experiments to establish which [tPA] would allow lysis within an hour under normal conditions. The ROTEM traces for each of the tPA concentrations used are shown in different colours, indicated above.

2.10.2 FIBTEM tests

In FIBTEM experiments, coagulation is activated as in EXTEM (with tissue factor), but the fib-tem reagent contains cytochalasin D which blocks platelet activity. FIBTEM experiments were performed similarly to EXTEM experiments

described above, with cups receiving 20 µl ex-tem reagent and 20 µl fib-tem reagent (both ROTEM, Tem International GmBH), prior to the addition of Affimer protein in a total volume of 90 µl saline solution (0.9% NaCl). Finally, 210 µl whole blood was added, mixed once with the pipette, and then the experiment initiated. FIBTEM tests were run for 1 h at 37 °C.

2.10.3 ROTEM parameters

During clot formation and lysis, multiple parameters are calculated from the measurements taken by the ROTEM delta. The ROTEM-calculated parameters clotting time (CT), clot formation time (CFT), and maximum clot firmness (MCF) were used to define clotting in the presence of Affimer proteins. CT is the time from the start of the test until a clot firmness amplitude of 2 mm is reached and reflects the speed of thrombin generation [322]. CFT is the time between 2 mm amplitude and 20 mm amplitude, and is largely influenced by thrombin generation, platelet count and function as well as fibrinogen concentration and fibrin polymerisation [322]. MCF is the maximum amplitude (in mm) reached by the clot during a test, and reflects the mechanical strength of the clot (Figure 2-4). MCF is influenced by stabilisation of the clot by fibrin polymerisation, FXIII activity, platelet count and platelet function [322]. Percentage lysis of clots was calculated from raw data extracted from the ROTEM. Lysis time was defined as the time from MCF to time of 50% reduction in MCF.

2.11 Platelet studies in whole blood

2.11.1 Platelet activation assays

Platelet activation assays were performed by Dr Benjamin Spurgeon.

Platelet activation was measured by whole blood flow cytometry as previously described [323]. Whole blood samples were collected from three healthy volunteers as previously described (section 2.10). Blood samples were incubated with buffer only (PBS), scaffold or Affimer F5 protein (Affimer:fibrinogen molar ratio of 10:1). Samples were stimulated with 5 µM thrombin receptor activating peptide (TRAP) and stained with platelet marker CD42b-APC and activation marker CD62PPE (BD Biosciences, California, USA) for 20 min. Samples were run on a Beckman Coulter CytoFLEX RUO Flow Cytometer and analysed on

FlowJo (v10, FlowJo LLC, Oregon, US). Automatic compensation was performed with BD CompBeads (BD Biosciences, California, USA).

2.11.2 Aggregation in whole blood

Fladia Hawkins performed whole blood aggregation experiments using the Multiplate Analyser.

Blood was collected in Hirudin Tubes (Roche Diagnostics International Ltd, Rotkreuz, Switzerland) and whole blood aggregation was performed using the Multiplate analyser (Dynabyte medical, Munich, Germany). Whole blood samples were used from three healthy volunteers as described previously (section 2.10). Blood (300 µl) was diluted 1:2 (v/v) with saline solution (0.9% NaCl) containing Affimer protein (Affimer:fibrinogen molar ratio of 10:1) (600 µl total volume). This blood/Affimer/saline mix was then incubated for three min at 37 °C. Agonists adenosine-diphosphate (ADP) or collagen (ADPtest; COLtest, Roche Diagnostics International Ltd, Rotkreuz, Switzerland) (final concentrations of 6.5 µM and 3.2 µg/ml respectively) were then added. The area under the aggregation curve and maximum aggregation were calculated.

2.12 Binding of Affimer proteins to fibrinogen and fibrin

2.12.1 ELISA binding assay

Nunc-Immuno Maxisorp 96 well ELISA plates (Thermo Fisher Scientific, Loughborough, UK) were coated with 100 µl of 5 µg/ml IF-1 purified fibrinogen in 50 mM sodium carbonate pH 9.6 and incubated at 4°C overnight. Wells were washed five times with 300 µl TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20 v/v, pH 7.4) and blocked with 300 µl Tris-buffered saline (TBS) containing 3% (w/v) BSA for 90 min at 37 °C. After being washed (as before), a dilution series of Affimer protein in 1% BSA TBST was added to the wells in a volume of 100 µl in duplicate and incubated for 1 h at 37 °C. The wells were washed again with TBST and incubated with 100 µl mouse anti-polyhistidine antibody (Roche, Welwyn Garden City, UK) diluted 1/1000 in 1% BSA TBST for 1 h at 37 °C, before washing five times with TBST as before. Wells were incubated with 100 µl HRP-conjugated rabbit anti-mouse antibody (Dako, Agilent Technologies; Stockport, UK) diluted 1/1000 in 1% BSA TBST for 1 h at 37 °C, before washing with TBST

and developed with 100 µl water-soluble substrate for HRP (OPD tablets, Thermo Fisher Scientific, Loughborough, UK). The reaction was stopped by the addition of 100 µl 1.5 M H_2SO_4 and the absorbance at 490 nm was read on a Multiskan Go plate reader.

To assess the interaction of Affimers with fibrin, an ELISA binding assay was performed as above with an additional step to convert the fibrinogen (coating the plate) to fibrin. After coating an ELISA plate overnight with fibrinogen, as above, plates were washed five times with 300 µl TBST before application of 100 µl of activation mix containing 0.5 U/ml thrombin and 2.5 mM $CaCl_2$. Plates were incubated for 1 h at room temperature on a microplate shaker, before washing five times with TBST as before. Plates were then blocked and the protocol completed as in the fibrinogen ELISA described above.

2.12.2 Biacore surface plasmon resonance (SPR) experiments

SPR experiments were run by Dr Nikoletta Pechlivani with the assistance of Dr Iain Manfield of the Centre for Biomolecular Interactions technology facility.

IF-1 purified human fibrinogen (5 µg/ml (14.7 nM) 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, Little Chalfont, UK). A reference surface was prepared by activating and deactivating the dextran without the addition of protein. 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. Affimer proteins were diluted in running buffer for experiments, and injected for 120 s at 50 µl/min in three replicate experiments (Affimer concentrations 6.125–800 nM). The surface was regenerated by flowing running buffer for 15 min sufficient to return signal 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.

An additional fibrin surface was prepared by injecting thrombin (1 U/ml) diluted in running buffer over immobilized fibrinogen at 2 µl/min for 45 min 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.

2.13 Plasmin degradation products assay

The following assay was performed to compare the fibrinogen degradation products of plasmin cleavage of clots with and without Affimer protein.

Eighteen clots were prepared with fibrinogen protein (Calbiochem, Merck Millipore, Watford, UK,) both with and without IF-1 purification, at 0.5 mg/ml (1.47 µM) in PB in a total volume of 90 µl. In each experiment, nine of the clots contained Affimer protein in 90 µl volume of PB at an Affimer:fibrinogen molar ratio of 10:1 and nine of the clots contained buffer only in place of Affimer. Clots were incubated at room temperature for 30 min, prior to addition of 30 µl of an activation mix containing 0.05 U/ml thrombin and 2.5 mM $CaCl_2$ and overnight incubation at 37 °C. After incubation, 30 µl of lysis mix containing 39 ng/ml (0.56 nM) tPA and 3 µg/ml (34 nM) plasminogen was added to all clots and the clots were returned to a 37 °C incubator. Clot lysis was halted with the addition of aprotinin (Sigma-Aldrich, Dorset, UK) to a final concentration of 2.6 µg/ml (400 nM) at nine different time points from 10 min to 120 min, and after overnight lysis. After lysis of each clot was halted with aprotinin, the sample was spun in a microfuge for 12 s, to pellet any remaining clot and allow 100µl of the supernatant to be taken to be analysed by SDS-PAGE (as described in 2.9.1).

2.14 Plasmin and tPA activity assays

2.14.1 Plasmin generation assay

The rate of plasmin generation by tPA in the presence and absence of Affimer protein was performed according to a modified protocol by Bobbink et al. (1997) [324]. Assays were performed in 96 well clear bottom plates (Greiner Bio One International GmbH). Wells contained 0.5 mg/ml (1.47 µM) fibrinogen (Calbiochem, Merck Millipore, Watford, UK), with increasing doses of Affimer protein in a total volume of 65 µl of assay buffer (40 mM Tris-HCl, 75 mM NaCl, 0.01% Tween-20 v/v, pH 7.4). FXIII (Enzyme Research Laboratories, Swansea, UK) was added to the wells at 3.7 µg/ml (11.6 nM) and plasminogen (Enzyme Research Laboratories, Swansea, UK) at 8.3 µg/ml (90.2 nM) in a volume of 35 µl assay buffer, prior to the addition of 50 µl of an activation mix containing 0.5

U/ml thrombin and 2.5 mM calcium. The plate was then read for 15 min every 12 s at 37 °C at 340 nm on a Multiskan Go plate reader. After 15 min, 50 µl of lysis mix containing 25 ng/ml (0.36 nM) tPA and 0.8 mM chromogenic substrate S2251 (Chromogenix, Diapharma Group Inc., West Chester, US) was added in 50 µl of assay buffer. S2251 hydrolysis was monitored via kinetic absorbance readings at 405 nm every 30 s for 5 h at 37 °C.

2.14.2 Plasmin activity assay

This assay was performed to determine if Affimer proteins had a direct effect on plasmin activity. Assays were performed in 96 well clear bottom plates (Greiner Bio One International GmbH). Wells contained Affimer protein in assay buffer (40 mM Tris-HCl, 75 mM NaCl, 0.01% Tween-20 v/v, pH 7.4) with plasmin at either 30 µg/ml, 15 µg/ml, 7.5 µg/ml or 3.75 µg/ml (361, 181, 90, 45 nM) in a total volume of 150 µl (all concentrations final). Plates were incubated at 37 °C for 15 min, prior to the addition of 50 µl of a mix containing S2251 (0.8 mM, Chromogenix, Diapharma Group Inc., West Chester, US) to wells. S2251 hydrolysis was monitored via kinetic absorbance readings at 405 nm every 30 s for 2 h at 37 °C on a Multiskan Go plate reader.

2.14.3 tPA activity assay

This assay was performed to determine if Affimer proteins had a direct effect on tPA activity. Assays were performed in 96 well clear bottom plates (Greiner Bio One International GmbH), wells contained Affimer protein and tPA in 150 µl assay buffer (50 mM Tris-HCl, 130 mM NaCl, 0.01% Tween-20, pH 7.4). tPA was used at a range of concentrations, 160 ng/ml, 80 ng/ml, 40 ng/ml, 20 ng/ml and 10 ng/ml (2.29, 1.14, 0.57, 0.29, 0.14 nM) (all concentrations final). Plates were then incubated at 37 °C for 15 min, prior to addition of 50 µl assay buffer containing chromogenic substrate S2288 (Chromogenix, Diapharma Group Inc., West Chester, US) at 2 mM. S2288 hydrolysis was monitored via kinetic absorbance readings at 405nm every 30 s for 2 h at 37 °C on a Multiskan Go plate reader.

2.15 Plasminogen and tPA interaction with Affimer

Binding assays were performed to investigate whether Affimer protein interacted with plasminogen or tPA, or prevented these proteins from binding to fibrin(ogen).

2.15.1 ELISA assays to investigate interaction of tPA, plasminogen and Affimer protein with fibrin DD fragment

Nunc-Immuno MicroWell 96 well-plates (Thermo Scientific, Loughborough, UK) were coated with 100 µl of 5 µg/ml (31 nM) fibrin DD fragment (Quadratech Diagnostics, UK) in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) and incubated overnight at 4 °C. Wells were washed five times with 300 µl TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4), before blocking with TBS containing 3% (w/v) BSA for 90 min at 37 °C. After washing as before, a dilution series of Affimer protein (0-5000 nM), plasminogen (0-40 µg/ml, 0-435 nM) or tPA (0-40 µg/ml, 0-571 nM) in 1% BSA TBST was added to the wells in duplicate and incubated for 1 h at 37 °C. The wells were washed again with TBST before application of antibody to the plate. Affimer was detected with mouse anti-polyhistidine 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 described in section 2.12.1.

Competitive binding assays were performed to determine if Affimer protein could prevent binding of tPA or plasminogen to fibrin DD fragment. These assays were performed with a slight modification of the above protocol. A concentration series of Affimer protein (0-10,000 nM) was incubated with either 1.25 µg/ml (13.6 nM) plasminogen or 1.25 µg/ml (17.9 nM) tPA for 1 h in the wells of a DD fragment-coated plate. tPA or plasminogen binding to DD in the presence of Affimer protein was detected.

2.15.2 ELISA assays to investigate Affimer interaction with tPA and plasminogen

ELISAs were performed as above (2.15.1), except plates were coated with 5 µg/ml plasminogen (54 nM) or tPA (71 nM) instead of fibrin DD fragment. A concentration series of Affimer protein was incubated with the tPA or plasminogen coated plates, and Affimer detected as previously described.

2.16 Pull down assay

A pull down assay was developed for the purpose of confirming Affimer-fibrinogen binding in solution, and to investigate any other possible Affimer-plasma protein interactions.

2.16.1 Protocol optimisation

To optimise the pulldown protocol, two types of magnetic affinity-beads for isolation of His-tagged proteins were compared, HisPur Ni-NTA beads and Dynabeads His-Tag Isolation and Pulldown (both Thermo Fisher Scientific, Loughborough, UK). Experiments were performed with both types of bead to determine the optimal wash buffer and loading buffer compositions.

2.16.1.1 HisPur Ni-NTA beads

The tube containing the HisPur Ni-NTA beads was inverted to ensure slurry was thoroughly mixed prior to use followed by pipetting 40 µl of beads into 1.5 ml Eppendorf tubes on a magnetic rack, and the bead storage buffer was removed. Beads were washed three times with 160 µl wash/load buffer (WLB, 25 mM Tris, variable mM NaCl, 1% NP40, 5% glycerol, 20 mM imidazole), which was added to the beads and then vortexed, before aspirating the WLB. A total of 50 µg Affimer was added to one set of tubes in a volume of 400 µl WLB, and 400 µl WLB-only added to the other tubes. Tubes were vortexed for 10 s, and then mixed for 30 min on a carousel mixer at room temperature. Beads were washed four times with 500 µl WLB.12 µl normal pool plasma was added to both tubes in a volume of 400 µl WLB and 4 µl EDTA-free Halt Protease Inhibitor (Thermo Fisher Scientific, Loughborough, UK) prior to 2 h incubation at 4°C on a roller. Beads were washed with 500 µl WLB, and wash buffers monitored for protein content on the Nanodrop. Washes were stopped when the Nanodrop showed no more protein being washed from the beads. Proteins were eluted with the addition of 25 µl elution buffer (WLB with the addition of imidazole to 300 mM) and vortexing for 15 s, prior to centrifuging for 1 min at 700x g. Tubes were incubated for 15 min on a rotating platform before the eluted proteins were aspirated.

The wash/load buffer (WLB) for the above protocol was used for dilution of the Affimer before adding to the beads, for dilution of plasma, and for wash steps. There were four different variations of the WLB in this assay, which differed by

NaCl concentration. The WLBs contained 25 mM Tris, 1% NP40, 5% glycerol, 20 mM imidazole, and either 75 mM, 150 mM, 300 mM or 450 mM NaCl.

2.16.1.2 Dynabeads

Dynabeads (Thermo Fisher Scientific, Loughborough, UK) were vortexed for 30 s to ensure a homogenous slurry, before aliquoting 40 µl beads into 1.5 ml Eppendorf tubes and aspiration of the bead storage buffer. 50 µg Affimer was added to one set of the tubes in a volume of 700 µl binding buffer (BB, 25 mM Tris, 300 mM NaCl, 0.01% Tween-20 v/v, 20 mM imidazole) and the other tubes received 700 µl of BB only. Beads were placed on a carousel mixer for 10 min at room temperature before being washed four times with 300 µl wash buffer (WB, 25 mM Tris, variable mM NaCl, 0.01% Tween-20, 20 mM imidazole). Beads were then re-suspended in 700 µl BB containing 12 µl of normal pool plasma and 7 µl EDTA-free Halt Protease Inhibitor (Thermo Fisher Scientific, Loughborough, UK). Tubes were mixed by inversion prior to incubation for 30 min at room temperature on a carousel mixer. Beads were then washed with 300 µl WB and wash buffers monitored for protein content on the Nanodrop. Washes were stopped when the Nanodrop showed no more protein being washed from the beads. Proteins were eluted with 50 µl elution buffer (25 mM Tris, 300 mM Nacl, 0.01% Tween-20, 300 mM imidazole), which was added to the tubes and then incubated for 10 min on a roller at room temperature, prior to collection of the elution buffer.

In these Dynabead experiments, four different wash buffers were trialled, varying in their [NaCl]. Wash buffers were 25 mM Tris, 0.01% Tween 20, 20 mM imidazole and 300 mM, 600 mM, 900 mM or 1200 mM NaCl.

Comparing the two types of affinity bead

The elutions from both types of affinity beads were compared by analysing proteins by SDS-PAGE. 20 µl of each elution was mixed with 7 µl NuPAGE LDS sample buffer and 3 µl NuPAGE sample reducing agent (both Invitrogen, Paisley, UK) and heated for 10 min at 95 °C. The entire 30 µl sample was then loaded into a 10% Bis-Tris gel (Invitrogen, Paisley, UK) which was run with MOPs buffer (Invitrogen, Paisley, UK) for 50 min at 200 V. The gel was washed three times with ddH_2O, prior to staining for 1 h with GelCode Blue Gel Stain (Thermo Fisher

Scientific, Loughborough, UK). After de-staining overnight in ddH_2O, the gel was imaged using a G:Box Chemi XT4 imaging system (Syngene, Cambridge, UK).

The amount of background binding to the two types of beads was determined by measuring the protein content of the elutions from the buffer only control for the beads. This enabled calculation of background binding per mg of beads used.

2.16.2 Optimised Dynabead assay

Optimised Dynabead assays followed the protocol described in 2.16.1.2. Binding buffer composition was 25 mM Tris, 300 mM NaCl, 0.01% Tween-20 v/v, 20 mM imidazole. Wash buffer was 25 mM Tris, 1M NaCl, 0.01% Tween-20, 20 mM imidazole. Elution buffer was 25 mM Tris, 300 mM Nacl, 0.01% Tween-20, 300 mM imidazole.

2.16.2.1 Pull down assays in afibrinogenemic plasma

To assess the ability of Affimers to interact with plasma proteins in the absence of fibrinogen, the above protocol for pull downs with Dynabeads was followed (section 2.16.2), performed with both normal pool plasma and pooled plasma from three afibrinogenemic patients. Afibrinogenemia patient plasma samples were provided by Dr Alessandro Casini (University of Geneva). Informed written consent was obtained from each volunteer in accordance with the declaration of Helsinki. Ethical approval was obtained from the University Hospitals of Geneva and Faculty of Medicine review board.

2.16.3 Pull down assays to find Affimer-fibrinogen interaction sites

Pull down assays were performed following a modified method of Zamolodchikov et al. (2016) [325] to locate Affimer F5-fibrinogen interaction sites. Affimer F5 (50 µg) or buffer only control was incubated with 0.5 mg/ml fibrinogen (Calbiochem, Merck Millipore, Watford, UK) in a total volume of 500 µl binding buffer (50 mM Tris, 100 mM NaCl, 0.01% Tween-20 v/v) for 30 min at room temperature. After incubation, 50 µl of a lysis mix containing 3 µg/ml plasmin was added to each tube, mixed, and incubated for 2 h at 37 °C. After lysis, aprotinin (Sigma-Aldrich, Dorset, UK) was added to each tube in 15 µl, to a final concentration of 400 mM to prevent any further lysis of fibrinogen. Imidazole was added to each sample to a final concentration of 20 mM, before the addition of 40 µl Dynabeads to each

tube. Tubes were mixed on a carousel mixer for 45 min at room temperature. Beads were then washed five times with 500 µl wash buffer (50 mM Tris, 500 mM NaCl, 0.01% Tween-20 v/v, 20 mM imidazole) on a magnetic rack, prior to elution of proteins with 30 µl elution buffer (50 mM Tris, 100 mM NaCl, 0.01% Tween-20 v/v, 300 mM imidazole). A total of 20 µl elutions were analysed by SDS-PAGE as previously described (section 2.9.1). Elutions were also analysed by SDS-PAGE under non-reducing conditions, which differs from the previously described method in that samples do not receive sample reducing agent, and are not heated prior to loading onto gels.

A variation of this experiment was also performed in which fibrinogen was digested with plasmin for 2 h prior to incubation with Affimer F5, and pull down as above.

To identify Affimer-binding fibrinogen degradation products, gel bands were sent for identification by mass spectrometry by Dr Mike Deery at the Cambridge Centre for Proteomics at the University of Cambridge.

2.17 Mass spectrometry

Mass spectrometry (liquid chromatography tandem mass spectrometry, LC-MS/MS) was performed by Dr Mike Deery and his team at the Cambridge Centre for Proteomics to identify protein bands from pull down elutions (section 2.16.3). These experiments were performed as previously described [326], briefly: gel bands of interest were cut into 1 mm^2 pieces, destained, reduced with dithiothreitol (DTT), alkylated with iodoacetamide and subjected to enzymatic digestion with trypsin overnight at 37 °C.

After trypsin digestion, the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis. All LC-MS/MS experiments were performed using nanoAcquity ultra-performance liquid chromatography (UPLC) (Waters Corp., Milford, MA) system and a linear trap quadrupole (LTQ) Orbitrap Velos hybrid ion trap mass spectrometer (Thermo Scientific, Waltham, MA).

Separation of peptides was performed by reverse-phase chromatography using a Waters reverse-phase nano column. The LC eluant was sprayed into the mass spectrometer by means of a standard Thermo Scientific nanospray source. All *m/z* values of eluting ions were measured in the Orbitrap Velos mass analyser. Post-run, the data was processed using Protein Discoverer (version 2.1., Thermo Fisher). Briefly, all MS/MS data were converted to mgf files and the files were then submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against a customised database containing human fibrinogen (alpha, beta and gamma chains) and common contaminant sequences (115 sequences, 38274 residues; http://www.thegpm.org/crap/).

2.18 Molecular modelling

Molecular modelling was performed by Dr Katie Simmons.

2.18.1 Fibrinogen fragment D and fibrin DD fragment

A homology model of Affimer F5 was created using I-TASSER and the Maestro graphical user interface to check the validity of the model produced [327]. The published crystal structure of the Affimer scaffold (PDB ID 4N6T) was used as a template to create a model of Affimer F5 [289]. Docking of this F5 model to the structure of fibrinogen fragment D (PDB ID 1FZA) [328] was carried out using AutoDock 4.2 [329]. A total of 100 docking iterations were calculated for each predicted site using a Lamarckian Genetic Algorithm. The resulting poses were clustered, based on a 2 Å root mean squared deviation. The cluster with the lowest energy conformation and also the most populated cluster pose were further examined using PyMOL [330, 331]. The fibrinogen residues within 4 Å of Affimer F5 variable region loops were examined in the structure of the DD fragment from fibrin (PDB ID 1FZC) [328], to determine if the residues remained accessible on conversion of fibrinogen to fibrin.

2.18.2 Affimer variable region peptides

The variable regions of Affimers B3 and F5 were modelled as two 9 amino acid peptides using the Maestro graphical user interface, and energy minimised using the OPLS3 force-field in a water environment [332]. The structures adopted by the 9 amino acid peptides was compared with the structure of the loops when part of the Affimer scaffold. Modelling of Affimer F5 is described above, modelling

of Affimer B3 was performed similarly, but using the crystal structure of Stefin A (PDB ID 3KFQ) as a template for B3.

2.19 Preparation of an affinity column with Affimer A2

Work described in relation to affinity purification was performed in collaboration with other members of the group, namely Fladia Hawkins, Dr Nikoletta Pechlivani, and Dr Ramsah Cheah.

SulfoLink Kit (Thermo Fisher Scientific, Loughborough, UK) was used for preparation of a column packed with resin covalently bound to Affimer A2 via the Affimer's C-terminal cysteine. Affimer A2 sample was first reduced with 2-mercaptoethylamine (2-MEA) to ensure cysteines were in a reduced state. Approximately 10 mg of Affimer A2 in sample preparation buffer (0.1 M sodium phosphate, 5 mM EDTA-Na, pH 6.5) was added to 6 mg of 2-MEA and incubated for 90 min at 37 °C, before being cooled to room temperature. The sample was then run through a desalting column to remove excess reductant from the sample and eluted in coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5). To covalently link the reduced Affimer A2 protein to the coupling gel (for packing of a column), 2 ml of coupling gel was pipetted into a column and the storage buffer removed. The column was equilibrated with 8 ml coupling buffer, before applying the reduced Affimer A2 sample and incubating for 15 min at room temperature with end over end mixing, followed by an additional 30 min without mixing. The column was washed with 6 ml coupling buffer before blocking the non-specific binding sites on the gel. Non-specific binding sites were blocked with application of a solution containing 15.8 mg L-cysteine-HCl in 2 ml coupling buffer (0.05 M cysteine final concentration) and incubating for 15 min with mixing at room temperature, followed by an additional 30 min incubation without mixing. The column was washed several times with wash buffer (1 M NaCl, 0.05% NaN_3), before washing with 4 ml PB. Column was stored at 4°C when not in use with 2 ml PB containing 0.05% sodium azide.

2.19.1 Affinity column for the purification of fibrinogen from commercial preparations and from plasma

For affinity purification of fibrinogen using the Affimer column described above, the following protocol was used. The column was used for the purification of

fibrinogen from a commercially available preparation (Calbiochem, Merck Millipore, Watford, UK) and from normal pool plasma.

The affinity column was allowed to come to room temperature before use, and the storage solution removed. The column was equilibrated by washing with 6 ml PB. Normal pool plasma was diluted 1:1 with PB, and approximately 1.8 mg fibrinogen was loaded onto the column in a volume of 1.5 ml. Calbiochem fibrinogen was diluted in PB so that approximately 2.5 mg fibrinogen was loaded onto the column in a volume of 1.5 ml. After loading of fibrinogen samples (Calbiochem or normal pool plasma), 1 ml PB was added to the column, and the column incubated for 1 h at room temperature. The column was then washed with 12 ml PB prior to elution of bound protein with elution buffer containing 50 mM glycine with either 50 mM NaCl (Calbiochem fibrinogen experiment) or 150 mM NaCl (plasma experiment). Fractions of 1 ml were collected and neutralised with 100 µl 1 M Tris, pH 7.5. The column was regenerated after use by first washing with 16 ml PBS to remove any residual protein and reactivate the gel. 8 ml PB containing 0.05% sodium azide were then run through the column prior to storage of the column with 2 ml PB containing 0.05% sodium azide at 4°C. Elutions were buffer exchanged into PB using desalting columns (Zeba spin, Thermo Fisher Scientific, Loughborough, UK), before running elutions on reducing gels as previously described in section 2.9.1. Approximate quantity of protein in each elution was determined by Nanodrop using fibrinogen extinction coefficient of 15.10. Functionality of eluted protein was determined using a turbidity and lysis (T&L) assay. Due to low yield of fibrinogen following affinity purification, the final fibrinogen concentration was 0.2 mg/ml (0.6 µM) in T&L assays. Fibrinogen was added to the wells of a 96 well plate, before addition of tPA (312 ng/ml, 4.5 nM), plasminogen (25 µg/ml, 272 nM) and thrombin (0.5 U/ml). Measurements were taken at 340 nm every 12 s for 1 h at 37 °C using a Multiskan Go plate reader.

2.20 The use of Affimer A2 for labelling of fibrin(ogen) in confocal microscopy

Work described related to use of Affimer A2 in confocal microscopy was designed by myself and Fraser Macrae, and confocal experiments performed by Fraser Macrae.

Free-flowing blood was taken from the antecubital vein of dysfibrinogenemia patients and hypofibrinogenemia patients and the blood centrifuged at 2,400x g for 20 min for platelet poor plasma (PPP). Dysfibrinogenemia and hypofibrinogenemia patient plasma samples were provided by Dr Alessandro Casini (University of Geneva). Informed written consent was obtained from each volunteer in accordance with the declaration of Helsinki. Ethical approval was obtained from the University Hospitals of Geneva and Faculty of Medicine review board.

To image the plasma samples, fibrinogen specific Affimer A2 was fluorescently labelled using an Alexa Fluor-488 protein labelling kit (as previously described in section 2.6.1). Fluorescently labelled Affimer was then added to patient plasma at 17.6 μM and incubated for 30 min. Clotting was initiated with 5 mM $CaCl_2$ and 0.5 U/ml of thrombin and a 30 μl drop of the mixture was immediately transferred to the centre of the well of an uncoated 8-well Ibidi slide (Thistle Scientific, UK). Clots were incubated for 4 h in a humidity chamber and were then imaged by LSCM as previously described in section 2.6.2. Experiments were also performed in which fluorescently labelled fibrinogen (Alexa Fluor-594, Invitrogen, Paisley, UK) was added to normal pool plasma with and without the labelled Affimer, to compare clot structure and fluorescent coverage of fibrin with conventionally used labelled fibrinogen. These samples were pipetted into the wells of a 6 channel Ibidi slide (Thistle Scientific, UK), and left for 4 h in a humidity chamber before imaging as previously described.

2.21 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 and statistical significance accepted at $p<0.05$. Distribution of data was tested using the Shapiro Wilk normality test. For data sets comprising three or more groups, the statistical significance between different groups was evaluated using one-way ANOVA followed by Dunnett's multiple comparisons test (for parametric data). For comparison of paired groups (three or more) data sets, repeated measures (RM) one-way ANOVA followed by Dunnett's multiple comparison test was performed (parametric data) or Friedman test followed by Dunn's multiple comparison test (non-parametric data).

For comparing two groups of paired data sets, statistical significance was determined using two-tailed, paired student t-test (parametric data) or two-tailed Wilcoxon matched-pairs signed rank test (non-parametric data), whereas unpaired data sets were compared using unpaired student t-test (parametric) or Mann-Whitney test (non-parametric). Unless otherwise stated the data are presented as mean±SD.

Chapter 3 Isolation of type II fibrinogen-binding Affimers

3.1 Introduction

Collaborators from the Astbury Centre for Structural Molecular Biology at the University of Leeds developed a small engineered protein scaffold called Affimer, which can be used for the study of protein-protein interactions [297]. Affimers exist in large phage display libraries comprising billions of clones, in which the Affimer scaffold constrains randomly generated variable region peptide sequences [289]. This library of Affimers was screened against fibrinogen, with the aim of isolating Affimers capable of modulating fibrin clot properties in a fibrinogen-specific manner. It was hoped that Affimers would modulate the property of fibrin clot lysis without causing non-physiological structural changes. To enable characterisation of fibrinogen-binding Affimers, a commonly used, high throughput plate-based clotting assay was optimised in plasma and purified conditions. Some potential issues arising from the use of Affimers, such as a propensity to aggregate and effects of Affimer-protein modification were also investigated, ultimately leading to the identification of a single fibrinogen-binding Affimer for further characterisation.

3.2 Results

3.2.1 Isolation of fibrinogen-binding Affimers

A total of 96 Affimers were picked following four rounds of phage panning and tested in an ELISA to confirm binding to fibrinogen (Figure 3-1). A total of 62 were chosen for DNA sequencing (denoted by the red lines below the well number in Figure 3-1) which identified 8 unique Affimer sequences.

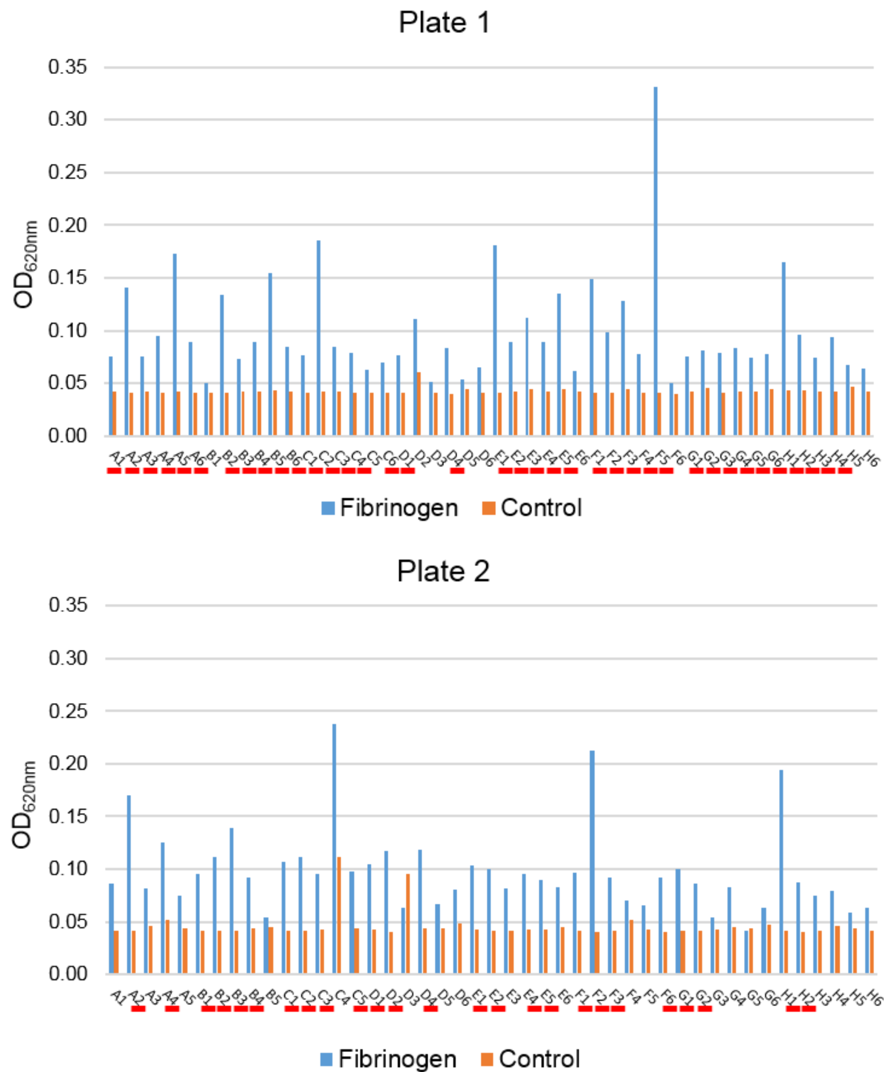


Figure 3-1 Selection of fibrinogen-binding Affimers by phage ELISA. A total of 96 phage clones were added to either biotinylated fibrinogen immobilised on streptavidin coated wells (blue bars) or control wells which did not contain fibrinogen (orange bars), across two 96 well plates. After incubation, wells were extensively washed and developed by the addition of an HRP conjugated anti-phage antibody to identify positive binders. The red lines indicate the 62 clones chosen for DNA sequencing.

These 8 Affimers were subsequently subcloned from their phagemid vector into pET11 vector (Figure 3-2A,B,C), expressed in *E. coli* and the Affimer proteins purified. Of the 8 fibrinogen-binding Affimers, 7 were successfully subcloned. Affimer B3 was found to have a single nucleotide mutation in the scaffold coding region of the phagemid vector, changing GAC (aspartic acid) to GGC (glycine) (Figure 3-2D) and therefore failed to express in *E. coli*. Control Affimers scaffold (lacking variable region inserts) and an Affimer against an irrelevant protein (anti-SUMO Affimer [333]) were also grown and have been used in experiments as negative controls.

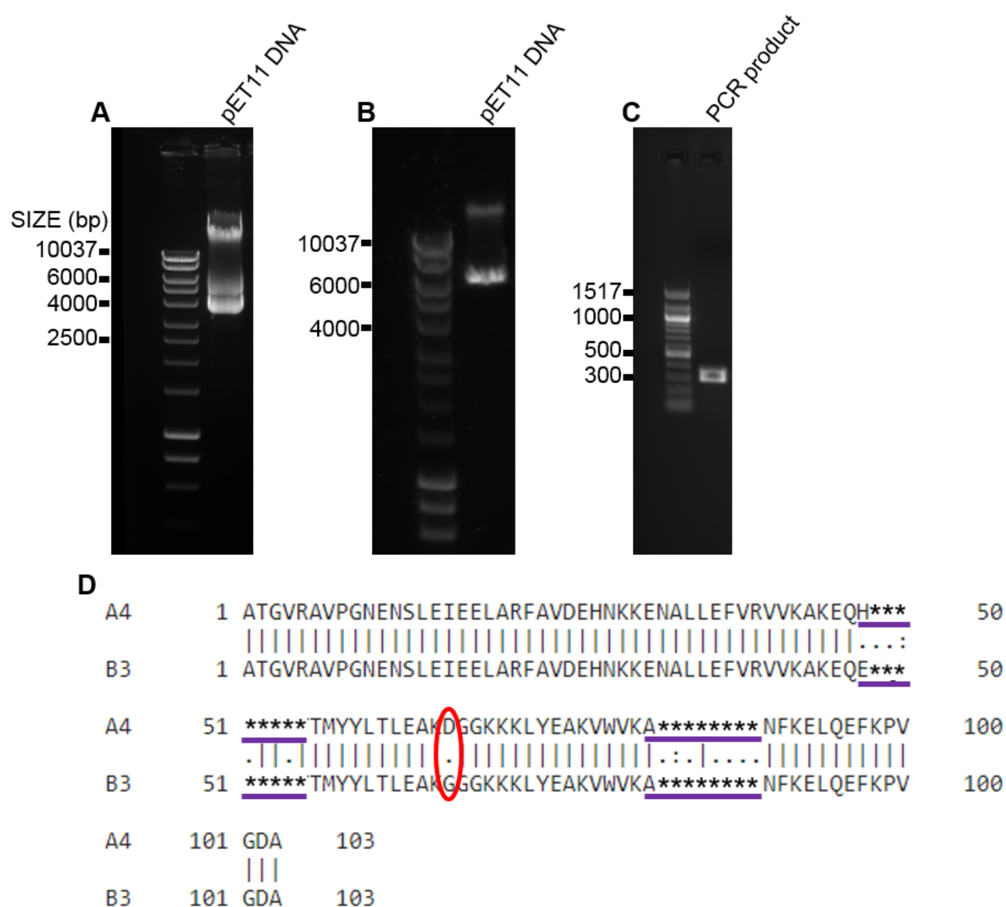


Figure 3-2 Affimer protein production. A total of 8 unique Affimer sequences were identified following DNA sequencing of 62 fibrinogen binders isolated from phage panning. **A.** For subcloning of the Affimers from the phagemid vector into the pET11 vector for protein production, a large quantity of pET11 was grown following a maxi-prep protocol and then digested with restriction enzymes. pET11 DNA run on a 1% agarose gel following maxi-prep. **B.** pET11 vector following restriction enzyme digest with Nhe1 and Not-1 on a 0.7% agarose gel. The digested vector (6000 bp) was cut from the gel and the DNA extracted. **C.** Polymerase chain reaction (PCR) products of amplified Affimer-coding region from the phagemid vector, run on 1% agarose gel (300 bp). **D.** Amino acid sequences of two Affimers (A4 and B3) are shown in a sequence alignment. The two variable regions of each Affimer are underlined in purple. Due to potential future patent considerations, only the first amino acids of the Affimer variable region loops are shown. A mutated amino acid in the scaffold region of B3 is circled in red, aspartic acid (D) is mutated to glycine (G) in B3.

3.2.2 Fibrinogen-binding ability of Affimers outside of the phage display system

The ability of the Affimers to bind to fibrinogen outside of the phage display system was compared with scaffold protein by ELISA. The OD for scaffold was 0.027±0.007, and was significantly greater for Affimers A2, A4, A5, B10, C2 and F5 that had OD 1.016±0.099 (P≤0.0001), 0.205±0.032 (P≤0.01), 0.554±0.025, 0.494±0.045, 4.320±0.057, 4.594±0.112 (P≤0.0001), respectively. These data demonstrated that the purified Affimers were capable of binding to non-biotinylated fibrinogen, outside of the phage display system. Affimer A1 and control Affimer anti-SUMO did not show significant fibrinogen binding (Figure 3-3).

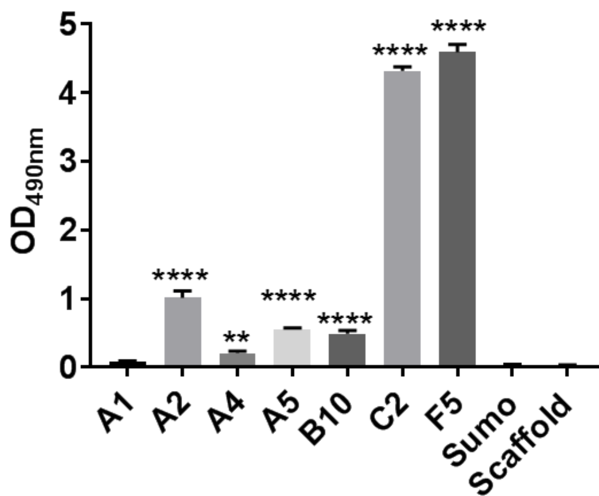


Figure 3-3 Affimer-fibrinogen binding. Wells of a 96 well plate were coated with Affimer protein. Controls included Affimer scaffold, and an anti-SUMO Affimer (sumo). Fibrinogen was incubated with the Affimer before washing and detection with a polyclonal anti-fibrinogen antibody. n=3, mean±SD of a single representative experiment is presented. Statistical analysis was performed using one-way ANOVA, **P≤0.01 or ****P≤0.0001 represents difference from scaffold control.

3.2.3 Optimisation of turbidimetric assays in plasma and purified systems

3.2.3.1 Plasma turbidimetric assay optimisation

Turbidimetric assays allow the assessment of clot formation and lysis in a high throughput format. Due to the existence of multiple protocols for this method, a range of conditions were tested in plasma and purified systems to determine those optimal for use in assays with Affimers. Initially, the effect of calcium and thrombin concentrations in turbidity assays in pooled human plasma were tested (Figure 3-4). Calcium at 7.5 mM and thrombin at 0.03 U/ml concentration resulted in the formation clots which did not proceed too quickly as to hinder the measurement of the lag time and showed a smooth clot formation profile. With these conditions, lag time was 7.5±1.0 min. The low thrombin concentration prolongs lag time, enabling analysis of samples in a 96 well plate, as described by others [319]. The use of these conditions in turbidimetric assays has also been shown to give good reproducibility [319]. Figure 3-5 shows the effect of altered calcium and thrombin concentrations on lysis assays in plasma, again, 7.5 mM calcium with 0.03 U/ml thrombin were the most favourable conditions. The tPA concentration was kept at 83 ng/ml (1.2 nM), in keeping with previously used protocols as it allows total clot lysis after approximately 1 h (66.7±3.5 min).

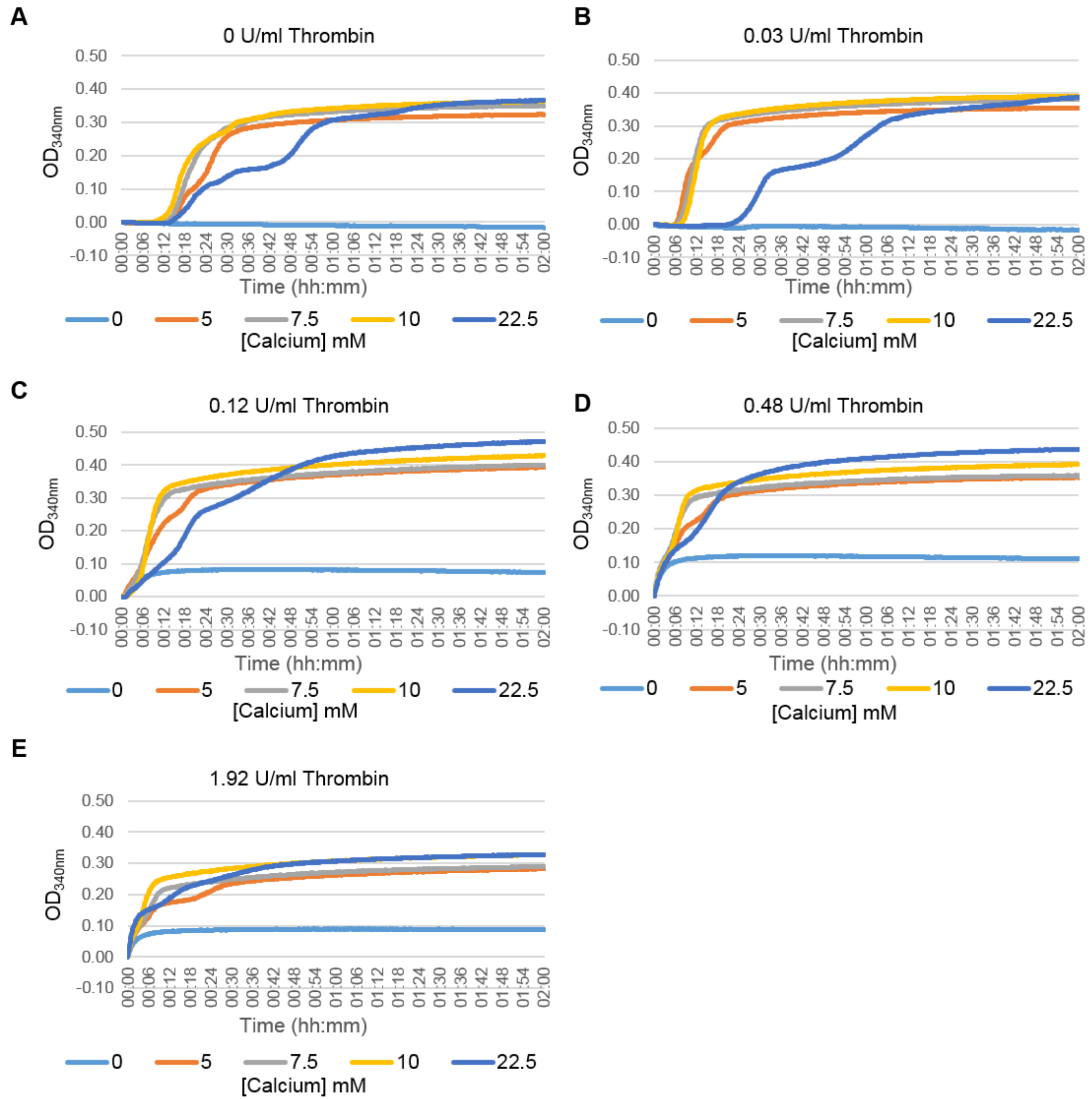


Figure 3-4 Plasma turbidity assay method optimisation. Pooled human plasma was added to the wells of a 96 well plate, and clotting initiated by the addition of thrombin and calcium. A concentration series of calcium (5 – 22.5 mM) was added to a reaction mix containing **A.** 0 U/ml, **B.** 0.03 U/ml, **C.** 0.12 U/ml, **D.** 0.48 U/ml and **E.** 1.92 U/ml of thrombin. Each reaction was measured in real time over a period of approximately 2 hr and plotted against optical density at 340 nm as a measure of clot formation. n=3, representative graphs shown.

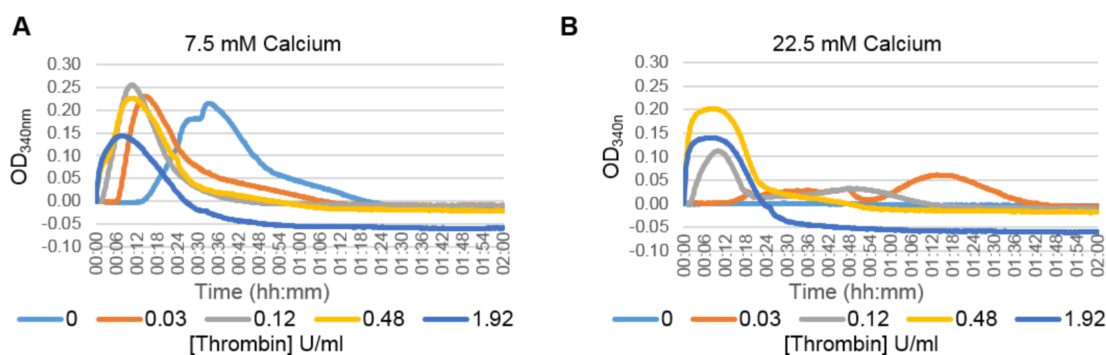


Figure 3-5 Plasma turbidity and lysis assay method optimisation. Pooled human plasma was added to the wells of a 96 well plate, and a lysis mix containing tPA was added. Clotting was initiated by the addition of thrombin and calcium. Calcium at **A.** 7.5 mM and **B.** 22.5 mM was used against five thrombin concentrations from 0.03 U/ml to 1.92 U/ml. Each reaction was measured in real time over a period of approximately 2 h and plotted against optical density at 340 nm as a measure of clot formation. n=3, representative graphs shown.

3.2.3.2 Purified turbidimetric assay optimisation

The standard turbidity and lysis protocol for purified fibrinogen systems used thrombin at 0.5 U/ml, plasminogen at 25 µg/ml (272 nM) and tPA at 312 ng/ml (4.5 nM), under which conditions clotting is initiated rapidly and changes to clot lysis profiles were difficult to observe (Figure 3-6A). Figure 3-6 shows the clotting/lysis profiles when a single Affimer was used in both the standard purified turbidimetric protocol (Figure 3-6A) and one in which thrombin, plasminogen and tPA concentrations were reduced (Figure 3-6B). These data demonstrate that at the higher thrombin concentration of 0.5 U/ml, clotting initiated rapidly, with no observable lag time in the buffer only control, while at the reduced thrombin concentration of 0.05 U/ml, lag time was prolonged to 3.2±0.6 min. These data also show that at high concentrations of tPA and plasminogen, there was an inability to determine changes in clotting/lysis characteristics in the presence of an Affimer protein.

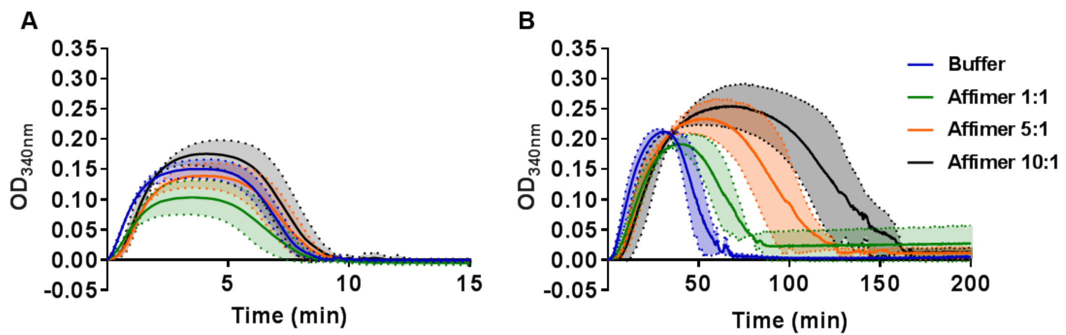


Figure 3-6 Comparison of high vs low thrombin/tPA/plasminogen concentration in purified turbidimetric assays. Fibrinogen was added to the wells of a 96 well plate with increasing concentration of an Affimer protein (Affimer:fibrinogen molar ratio of 1:1, 5:1 and 10:1). An activation mix containing thrombin and calcium was added to initiate clotting, and a lysis mix containing tPA and plasminogen to allow lysis to proceed. **A.** Thrombin, tPA and plasminogen concentrations of 0.5 U/ml, 312 ng/ml and 25 µg/ml, respectively. **B.** The same assay was performed with thrombin at 0.05 U/ml and an 8-fold dilution of tPA and plasminogen. Each reaction was measured over a period of approximately 3 h and plotted against optical density at 340 nm as a measure of clot formation/lysis. n=3, mean±SD.

In order to determine which tPA and plasminogen concentrations to use in purified turbidimetric assays, optimisation experiments were performed in which tPA concentration was varied from 0-312 ng/ml (0-4.5 nM) and plasminogen from 0-25 µg/ml (0-272 nM) in two-fold dilution series (Figure 3-7). An 8-fold dilution of tPA and plasminogen compared with the standard assay, at 39 ng/ml (0.56 nM) and 3.12 µg/ml (34 nM) respectively, was chosen, which provided time to full lysis that was similar to plasma assays (71.3±22.3 min), while maintaining ratio of plasminogen/tPA concentration used previously in our laboratory.

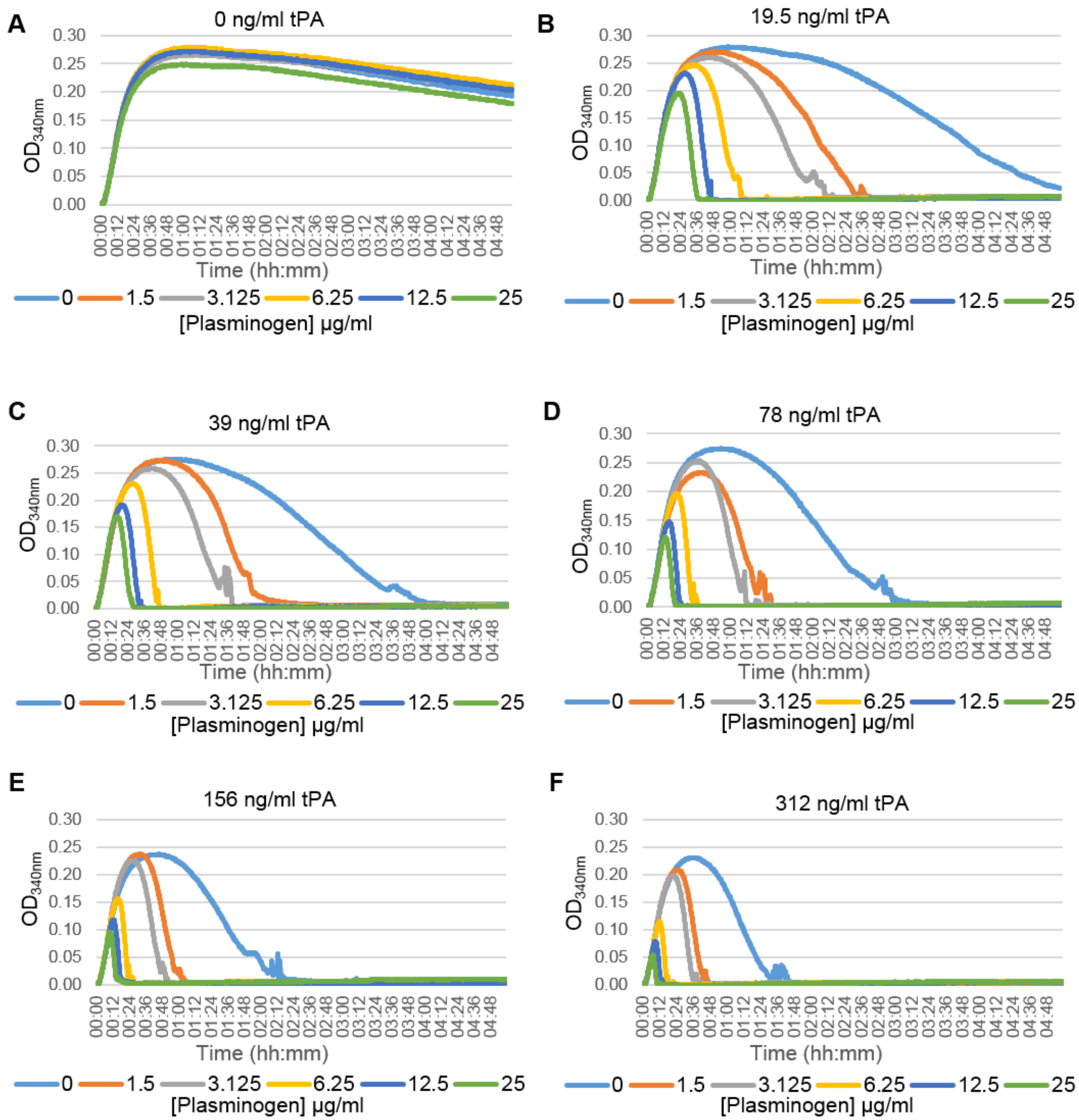


Figure 3-7 Purified turbidity and lysis assay method optimisation. Fibrinogen was added to the wells of a 96 well plate, and an activation mix containing thrombin and calcium initiated clotting. Lysis mix contained plasminogen (0-25 µg/ml) in a 2 fold dilution series and was tested against tPA at **A.** 0 ng/ml, **B.** 19.5 ng/ml, **C.** 39 ng/ml, **D.** 78 ng/ml, **E.** 156 ng/ml **F.** 312 ng/ml. Each reaction was measured in real time over a period of approximately 5 h and plotted against optical density at 340 nm as a measure of clot formation. n=3, representative graphs shown.

3.2.4 The effect of fibrinogen-binding Affimers on clot formation and lysis in plasma

The 7 fibrinogen-binding Affimers with distinct sequences were used in turbidimetric assays in plasma to assess their effect on clot properties (Figure 3-8). Affimers A2 and B10 significantly prolonged lag time compared with buffer only control, whereas C2 and F5 shortened lag time. Affimers A2, A4, B10, C2 and F5 significantly reduced maximum OD. Lysis time was increased by A2, B10, C2 and F5, however, only the latter two demonstrated statistical significance. These results are summarised in Table 3-1.

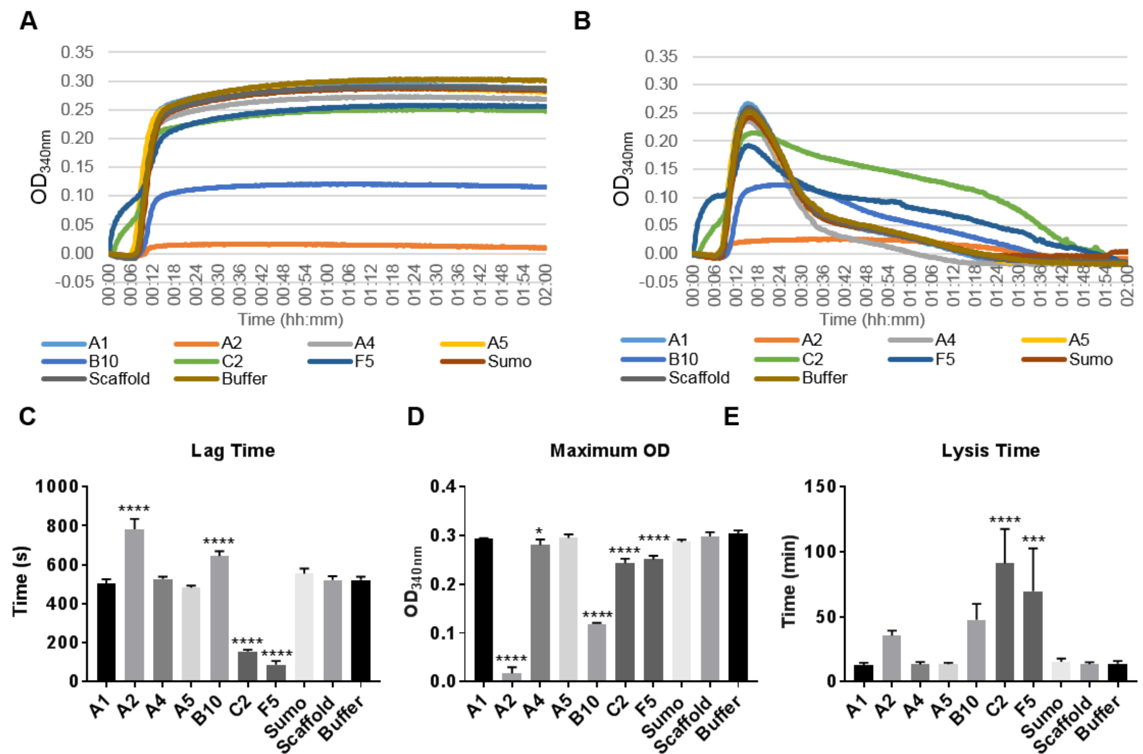


Figure 3-8 Effect of Affimers on clot formation and lysis in plasma. Pooled human plasma was pre-incubated with Affimers at 5:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) or thrombin, calcium and tPA (turbidity and lysis assays). Control Affimer proteins were Affimer scaffold, and an anti-SUMO (sumo) Affimer. **A.** Representative turbidity curves of Affimers in plasma. **B.** Representative turbidity and lysis curves in plasma. **C.** Lag Time **D.** Maximum OD. **E.** Lysis time. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, $^*P \leq 0.05$, $^{***}P \leq 0.001$, or $^{****}P \leq 0.0001$ represents difference from buffer only control.

Affimer	Lag time (s, mean±SD)	Maximum OD (AU, mean±SD)	Lysis time (min, mean±SD)
A1	505±20	0.2931±0.0016	12.9±1.7
A2	782±54****	0.0171±0.0128****	35.4±3.9
A4	527±12	0.2806±0.0113*	13.3±1.9
A5	483±10	0.2953±0.0072	13.5±1.1
B10	645±25****	0.1187±0.0021****	47.6±12.4
C2	155±9****	0.2436±0.0090****	91.0±26.6****
F5	87±25****	0.2510±0.0073****	69.7±32.8***
Anti-SUMO (sumo)	554±26	0.2870±0.0045	15.2±2.6
Scaffold	521±21	0.2981±0.0088	13.5±1.5
None (buffer only)	521±18	0.3038±0.0068	13.9±2.0

Table 3-1 The effects of fibrinogen-binding Affimers on clot parameters in plasma turbidimetric assays. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05, ***P≤0.001 or ****P≤0.0001 represents difference from buffer only control.

Turbidimetric assays were also performed with anti-SUMO and scaffold control Affimers to ensure that the presence of Affimer alone was not sufficient to cause disruption to clot formation and lysis and that the Affimer scaffold was not having an effect. No significant differences were found in plasma (Figure 3-9A) or purified (Figure 3-9B) systems when anti-SUMO and scaffold were added in increasing concentrations, when compared to buffer only control.

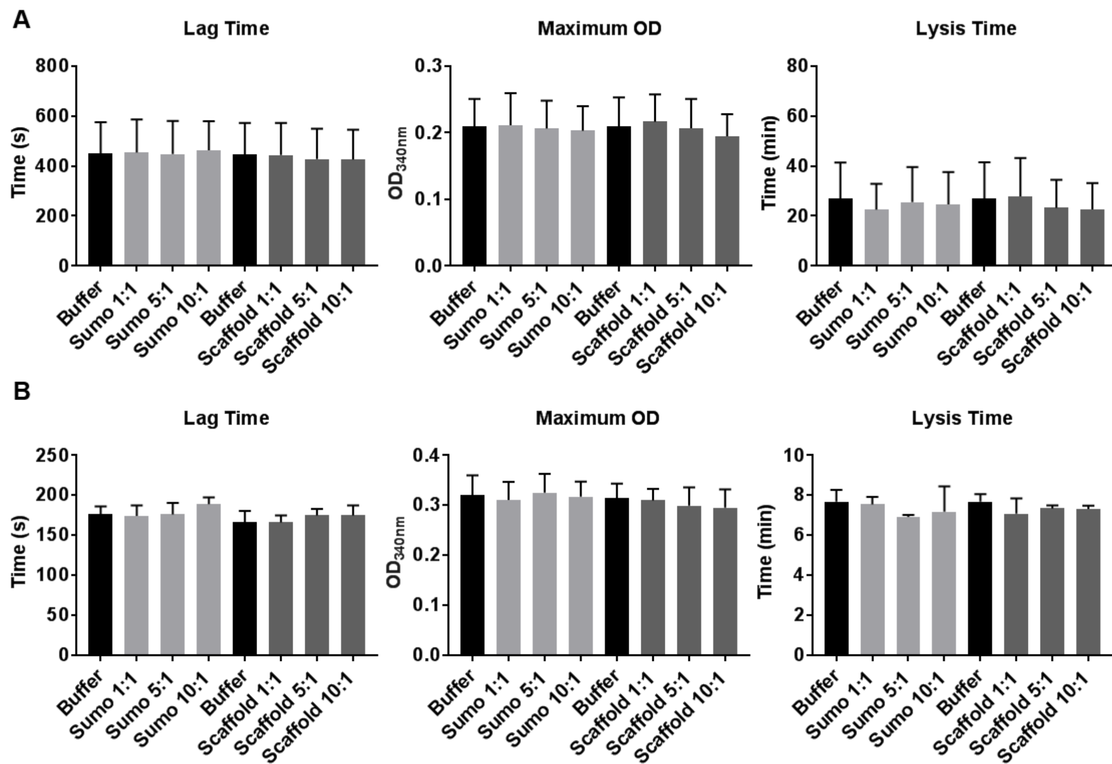


Figure 3-9 Effect of control Affimers scaffold and anti-SUMO (sumo) on clot formation and lysis in purified and plasma systems. Pooled human plasma or fibrinogen was pre-incubated with Affimers at 1:1, 5:1 and 10:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) and thrombin, calcium and tPA (turbidity and lysis assays). Buffer only samples contained no Affimer protein. **A.** (Left to right) lag time, maximum OD and lysis time of sumo and scaffold controls in a plasma system. **B.** (Left to right) lag time, maximum OD and lysis time of sumo and scaffold controls in a purified system. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, comparing each molar ratio to buffer only control.

3.2.5 The effect of Affimers on clot structure in plasma

Given that plasma clot final turbidity represents both fibre thickness and clot density, confocal microscopy was used to examine the effects of Affimers on clot structure in more detail (Figure 3-10A). Control Affimers anti-SUMO and scaffold caused no morphological changes to clot structure when compared with buffer control (Figure 3-10B). Affimers A2 and B10 caused an increase in average fibre count, from 27.3±4.1 fibres per 100 µm to 72.0±8.7 with A2 and 58.7±8.1 with B10 ($P \leq 0.0001$), in contrast to F5 which caused a decrease in fibre count to 16.9±3.9 ($P \leq 0.05$) (Figure 3-10C) and also produced a clot with irregularly arranged fibres. C2 caused morphological changes in clot structure which were not related to fibre count, clots appeared to be made up of more curled fibres and/or had more branch points (Figure 3-10A).

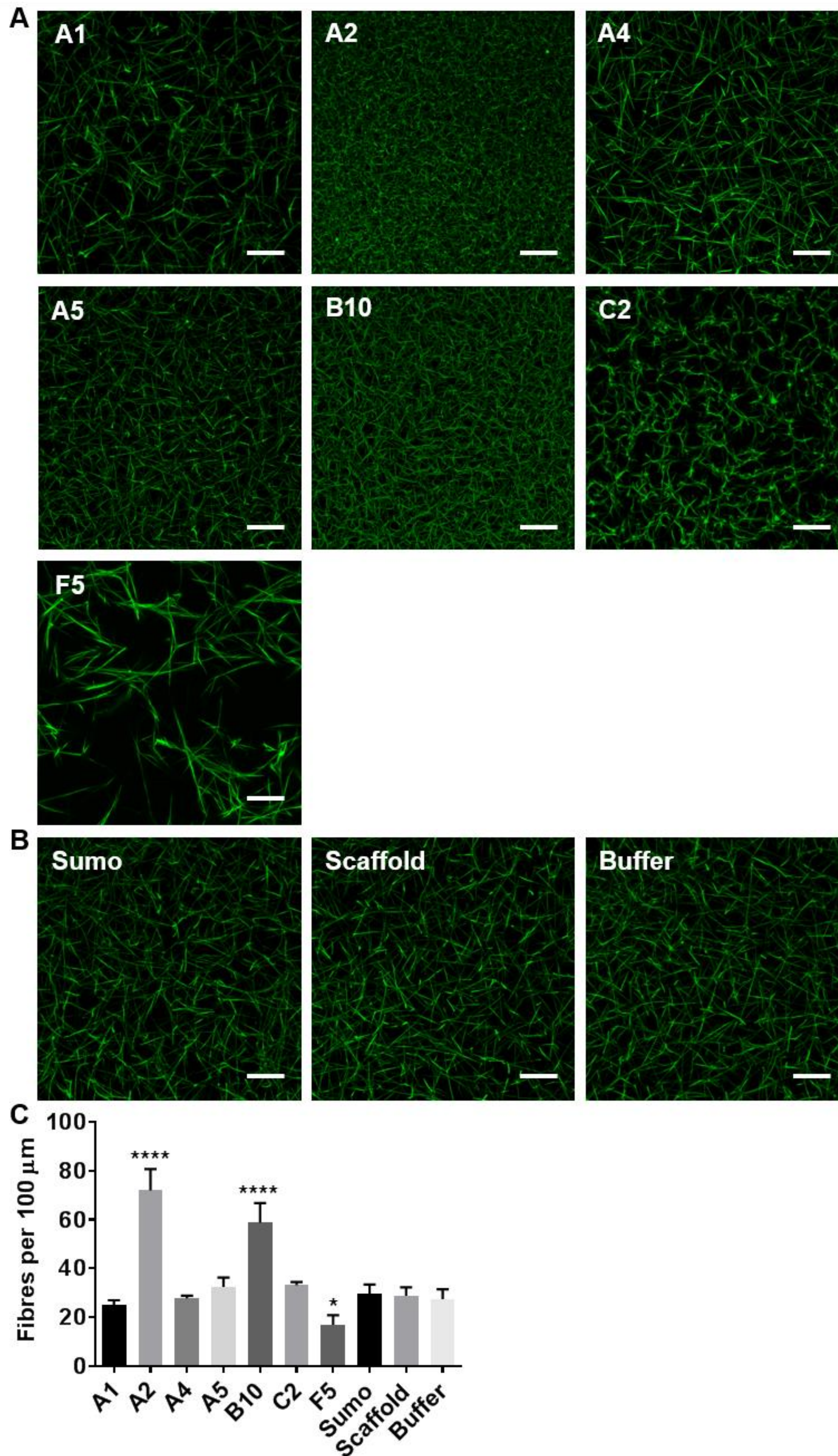


Figure 3-10 Effect of Affimers on clot structure in a plasma system. A. Confocal microscopy images of plasma clots formed in the presence of Affimers A1, A2, A4, A5, B10, C2, F5 at 5:1 Affimer:fibrinogen molar ratio. **B.** Controls were anti-SUMO (sumo), scaffold, and buffer (no Affimer protein). **C.** Average fibre count in confocal images. Two clots were made for each condition, and 3 images taken in different areas of each clot. Fibre density was determined by counting the number of fibres that cross 20 lines across each image, using ImageJ software. Data presented as mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05 or ****P≤0.0001 represents difference from buffer only control. Scale bar = 20µm.

Affimers A2 and B10 were chosen to study in more detail because of their less extreme effect on clot lysis in turbidimetric assays and clot structure in confocal microscopy when compared with C2 and F5.

3.2.6 Identification of high molecular weight products in Affimer protein samples

Purified Affimers were analysed by SDS-PAGE to assess purity and integrity of the protein. Some of the Affimers ran with high molecular weight (HMW) products as well as the expected band at ~15 kDa (representing monomeric Affimer protein) (Figure 3-11A).

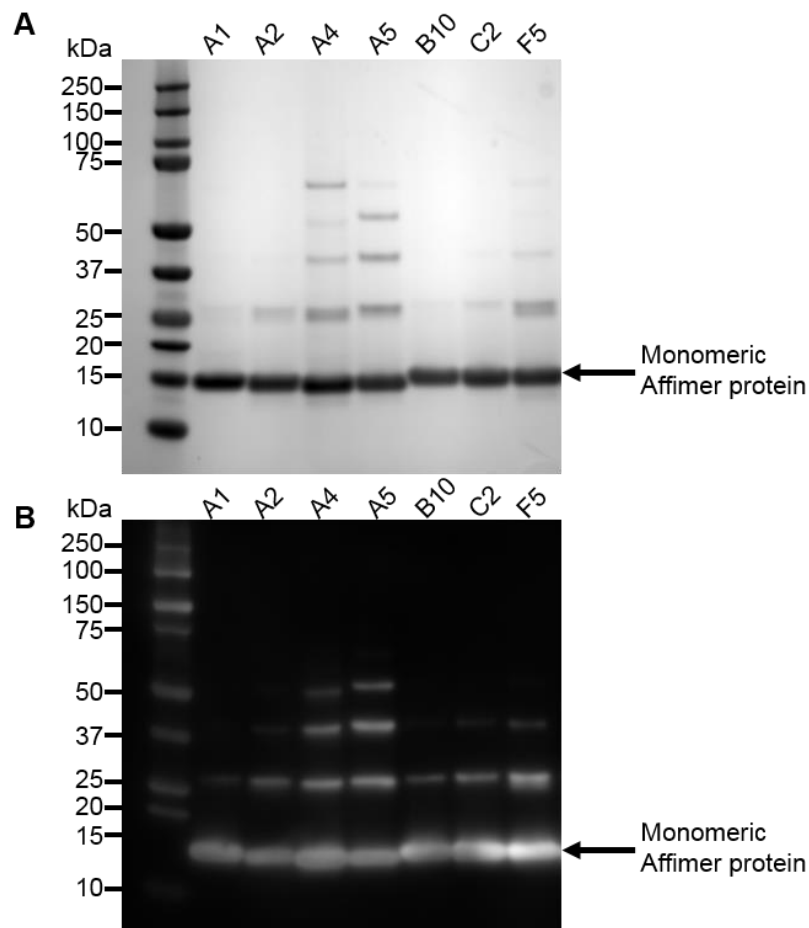


Figure 3-11 Identification of Affimer protein aggregates by SDS-PAGE and western blotting. Affimer protein samples were run on 4-12% Bis-Tris gels under reducing conditions. Gels were both stained to visualise protein and transferred to PVDF membrane to be blotted with an anti-polyhistidine antibody. **A.** Stained gel, Affimer protein present most abundantly in monomeric form as a band at ~15 kDa. **B.** Western blot using anti-polyhistidine antibody, detection of Affimer at ~15 kDa and as higher molecular weight protein aggregates.

The molecular weights of the HMW bands on the gel were multiples of Affimer protein molecular weights, suggesting they were multimeric Affimer complexes (Figure 3-11A). A western blot using an anti-polyhistidine antibody confirmed almost all of the HMW products as Affimer protein (Figure 3-11B). A single band of ~60 kDa was visible in the gel in the A4 protein sample, and not in the western blot. Size exclusion chromatography (SEC) was performed to purify the monomeric Affimer from the multimer complexes for A2, B10 and scaffold (data shown in Figure 3-12 for B10).

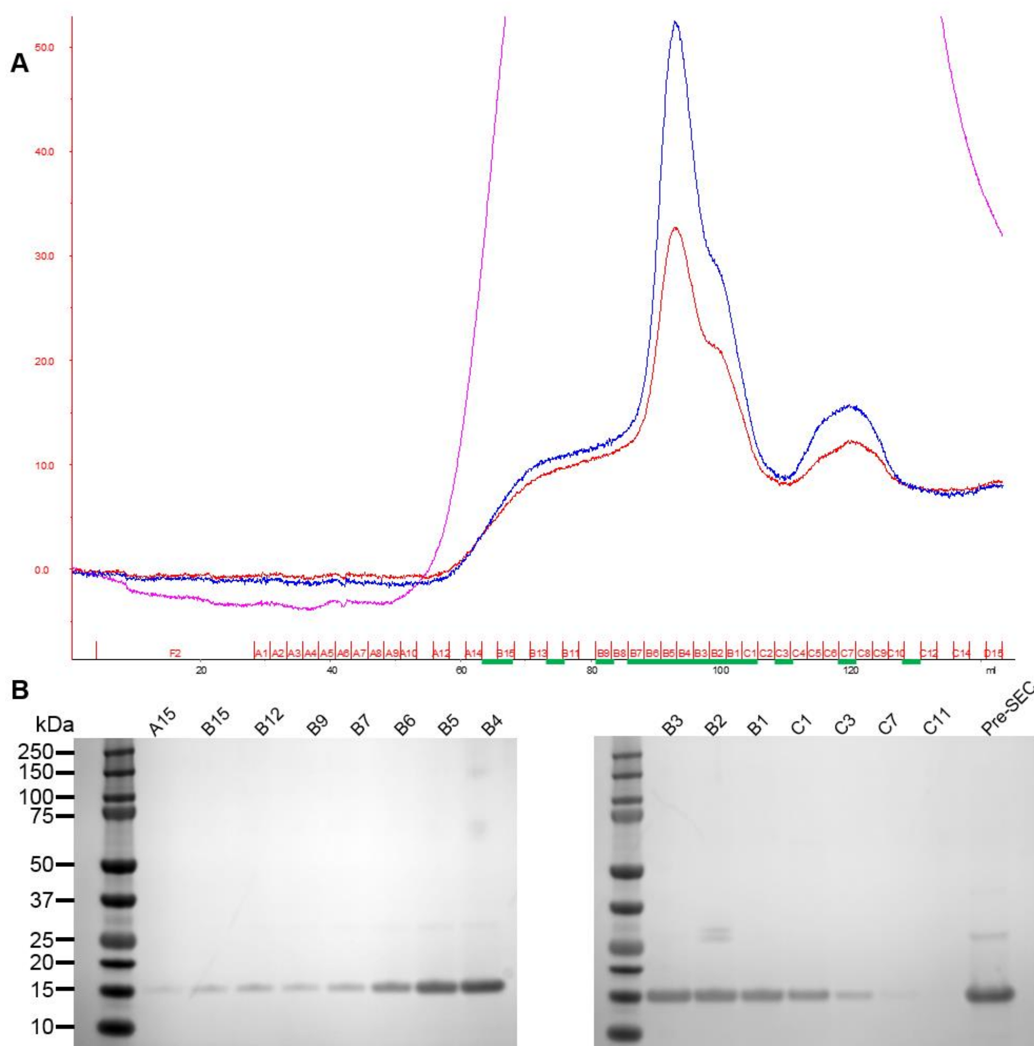


Figure 3-12 Size exclusion chromatography (SEC) to purify monomeric Affimer B10. Affimers A2, B10 and scaffold were purified by SEC, representative Affimer data shown. **A.** Trace from a size exclusion chromatography run with Affimer B10. Fractions run on gels are underlined in green on the x-axis. Peak traced with pink line is absorbance of fractions at 220 nm, red line at 260 nm and blue line at 280 nm. **B.** Fractions highlighted in green in (A) were analysed by SDS-PAGE. Fractions B6, B5 and B3 were pooled and concentrated for use.

3.2.7 Re-subcloning Affimers to remove C-terminal cysteine

Affimers were subcloned to contain a C-terminal cysteine to allow for targeted labelling of Affimer protein in downstream applications, however, there were concerns about the possible effect of the free cysteine on Affimer function in various assays (detailed in the discussion). Because of this, the 7 fibrinogen-binding Affimers were subcloned without the C-terminal cysteine, and tested in plasma turbidimetric assays (Figure 3-13A-E). Affimer F5 was the only Affimer to retain an effect on plasma clot lysis in turbidity and lysis assays, prolonging time to half lysis from 25.5±14.1 min to 99.9±20.0 min (P≤0.0001) (mean±SD) (Figure 3-13E). The effect of the 7 Affimers on clot structure was assessed using confocal microscopy, as before (Figure 3-14A-C). Without the C-terminal cysteine, none of the Affimers significantly altered average fibre count (Figure 3-14C). Because of these data, Affimer F5 was chosen to continue to study in more detail.

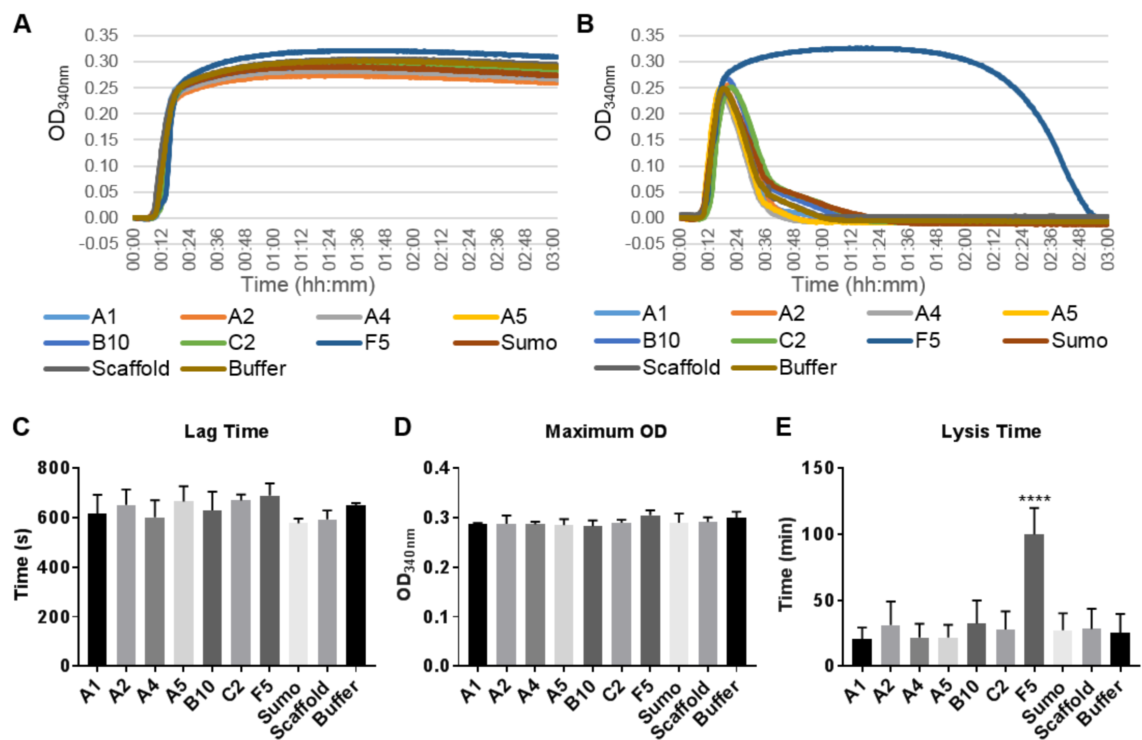


Figure 3-13 Effect of Affimers on clot formation and lysis in plasma, after removal of Affimer C-terminal cysteine. Pooled human plasma was pre-incubated with Affimers at 5:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) or thrombin, calcium and tPA (turbidity and lysis assays). **A.** Representative turbidity curves of Affimers in plasma. **B.** Representative turbidity and lysis curves of Affimers in plasma. **C.** Lag Time **D.** Maximum OD. **E.** Lysis time. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, ****P≤0.0001 represents difference from buffer only control.

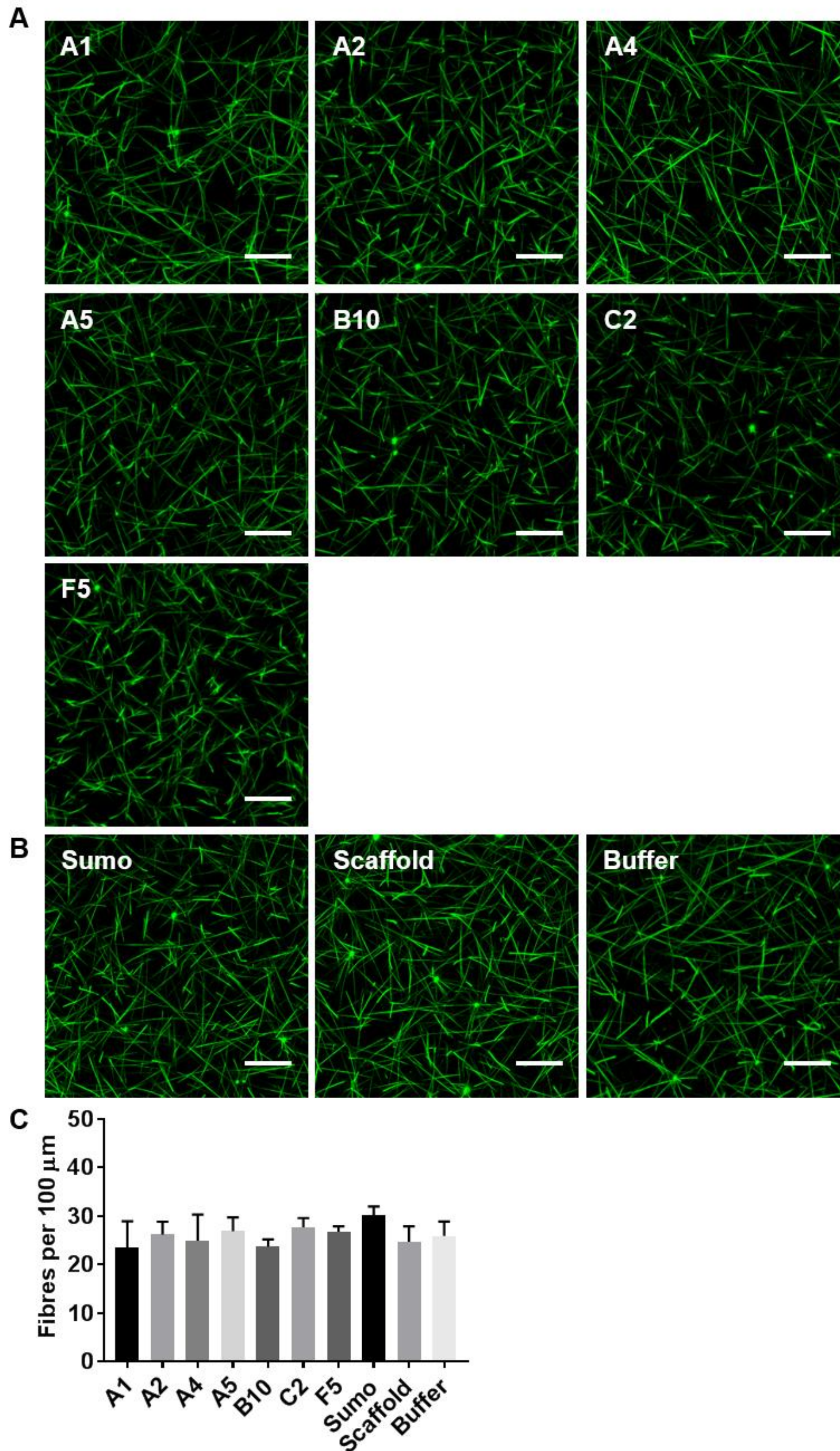


Figure 3-14 Effect of Affimers on clot structure in a plasma system, after removal of Affimer C-terminal cysteine. Confocal microscopy images of plasma clots formed in the presence of Affimers A1, A2, A4, A5, B10, C2, F5 at 5:1 Affimer:fibrinogen molar ratio. **B.** Controls were anti-SUMO (sumo), scaffold, and buffer (no Affimer protein). **C.** Average fibre count in confocal images. Two clots were made for each condition, and 3 images taken in different areas of each clot. Fibre density was determined by counting the number of fibres that cross 20 lines across each image, using ImageJ software. Data presented as mean±SD. Statistical analysis was performed using one-way ANOVA, comparing each Affimer to buffer only control. Scale bar = 20 µm.

3.3 Discussion

This chapter describes isolation of fibrinogen-binding Affimers and their characterisation, using optimised turbidity and lysis assays.

The scale and quality of the Affimer phage library permits rapid screening of a target protein with a large number of potential binding sequences constrained within the Affimer scaffold. Following three panning rounds, 8 unique Affimer sequences were identified, before the Affimer coding regions were subcloned into an expression vector to allow purification of the Affimer protein without the phage. This was done for two reasons: i) to rule out interference from the phage and ii) to allow for Affimer protein production in large quantities. One Affimer, termed B3, did not grow following transformation of the Affimer-containing vector into *E. coli*, likely due to the mutation of an amino acid in the Affimer scaffold as a result of substitution of a single nucleotide. Negatively charged aspartic acid at position 66 was replaced with a neutral glycine residue, a mutation which may have resulted in improper folding of the protein. The remaining 7 Affimers were purified and assessed for an ability to alter fibrin clot properties.

Of the 7 Affimers identified, 6 showed significant binding to fibrinogen outside of the phage display system, confirming an ability to bind non-biotinylated fibrinogen. Affimer protein A1 did not show significant binding to fibrinogen, suggesting that this Affimer may have been isolated during phage panning for an ability to bind to biotin. This is supported by sequence similarity of one of Affimer A1's variable regions with biotin-binding proteins - biotin protein ligase and biotin carboxylase from *Desulfovibrio aminophilus* and *Bradyrhizobium jicamae*, respectively. These sequence similarities were identified using a BLAST search (Basic Local Alignment Search Tool) with the Affimer variable regions as input against the online sequence database.

Following optimisation of turbidimetric assays, 3 of the 7 Affimers had no effect on lysis time in plasma, while 4 of the Affimers prolonged lysis time, and these changes were accompanied by alterations to lag time. 2 of the Affimers, A2 and B10 prolonged lag time, while C2 and F5 shortened it. All 4 of these Affimers caused a reduction in maximum OD, suggesting changes to the fibrin clot structure, which was confirmed by confocal microscopy. A2 and B10 caused the

formation of clots which were more dense when viewed under confocal microscopy, which fits with published data that clots formed of densely packed fibres typically take longer to lyse [137]. Affimers C2 and F5 caused formation of clots with altered fibrin morphology, appearing more curled/branched and with thicker fibres, respectively. It is possible that these changes in fibrin clot structure were responsible for the prolongation of lysis by these Affimers. On a single fibre level, thicker fibres take longer to lyse than thin ones [132] and more highly branched networks have been associated with slower lysis times [130, 137].

Before further work on the characterisation of Affimers could continue, the aggregation of Affimer proteins was investigated. Following Affimer protein production, protein integrity and purity were assessed by analysing the proteins using SDS-PAGE. Protein bands were present in the gels which were suspected multimers of Affimer proteins and western blotting with an anti-polyhistidine antibody strongly suggested this to be the case. Although the anti-polyhistidine antibody is not specific for Affimer protein, the combination of these factors point to an Affimer origin of the bands i) the molecular weights of the protein bands ii) recognition by the anti-polyhistidine antibody, which recognises epitopes of 6 consecutive histidines, as found in the Affimer his-tag iii) published instances of Affimer protein aggregation. Although formation of dimers by Affimer protein has been reported by others [298], aggregation was a concern due to the possibility that multiple species of Affimer could exist in a single sample, thereby resulting in unpredictable and inconsistent results in clotting assays. Protein bands present in Affimer A4 at around 60 kDa were not identified by the anti-polyhistidine antibody in a western blot, suggesting that they were not of Affimer protein origin. As described in later chapters, purification of Affimers from the bacterial system used for protein production can result in the contamination of Affimer samples with bacterial proteins. Since A4 was not an Affimer of interest, further purification of A4 was not pursued. However, Affimers A2 and B10 were subjected to SEC to purify the monomeric form of each Affimer protein, which displayed similar properties in turbidimetric assays as before SEC purification. This is perhaps because of all the Affimer samples investigated for multimer formation, Affimers A2 and B10 had relatively few additional protein bands corresponding to Affimer protein aggregates.

In addition to the presence of multimeric Affimer protein complexes, the impact of a C-terminal cysteine in the Affimer scaffold was investigated. Affimers were originally subcloned to contain a C-terminal cysteine to allow for their targeted modification in downstream applications. However, concerns arose in relation to the potential effects of free cysteines in the context of clotting. For example, in fibrinogen Dusart, covalent complexes have been observed between a free cysteine on fibrinogen and plasma protein albumin [213, 334]. Additionally, the presence of cysteines in the Affimer scaffold was likely a contributing factor to Affimer aggregation/formation of multimeric complexes. Therefore, the Affimers were subcloned again without the cysteine. Of the Affimers without a C-terminal cysteine, only Affimer F5 retained an effect on clot lysis in a turbidimetric assay, prolonging clot lysis by around 4-fold in a plasma system. Interestingly, none of the Affimers significantly altered fibrin fibre count, including Affimer F5, suggesting that the anti-fibrinolytic mechanism of F5 was not dependent on changes in fibrin clot structure, but related to interference with the efficiency of the fibrinolytic system.

Affimer F5 was chosen to study further, to characterise the mechanism by which the protein prolonged lysis without causing major structural changes to the fibrin network. An agent which stabilises the fibrin clot and delays its breakdown has potential therapeutic benefit in the context of bleeding following trauma, surgery, or that is associated with some coagulation factor deficiencies.

Chapter 4 Characterisation of type II fibrinogen-binding Affimer with anti-fibrinolytic properties

4.1 Introduction

The previous chapter was aimed at isolating fibrinogen-binding Affimers and investigating the use of Affimers in the context of clot formation and lysis. This included determination of the effect of the Affimers protein’s ability to form multimers, and the effect of cysteine-modification of the Affimer scaffold. In the previous chapter, Affimer F5 was shown to prolong fibrinolysis and thus has potential beneficial use in clinical conditions caused by excessive bleeding.

In this chapter the effects of Affimer F5 are investigated in a variety of systems, and a mechanism of action is proposed for the Affimer. The following data show that fibrinogen-binding Affimers represent a novel methodology for identification of protein-fibrinogen interactions or alternatively for hotspots on fibrinogen which are prime targets for modulation by small molecules. There is potential for clinical benefit in conditions characterised by bleeding caused by clot instability and enhanced fibrinolysis.

4.2 Results

4.2.1 F5 binds to fibrinogen and fibrin with high affinity

To define the binding of F5 to fibrinogen and fibrin a combination of SPR and plate-based ELISA assay were used. SPR experiments were performed by Dr Nikoletta Pechlivani and Dr Iain Manfield. ELISA assay showed the K_D of interaction for F5 with fibrinogen and fibrin to be 217.6 nM±47.8 nM and 273.6 nM±23.2 nM (mean±SD), respectively (Figure 4-1A,B). SPR experiments also demonstrated that Affimer F5 bound with similar affinity to both fibrinogen and fibrin. F5 bound immobilized fibrinogen with K_D value of 52±1.3 and to fibrin with a K_D value of 58±9.3 nM (Figure 4-1C,D). Affimer F5 demonstrated fast association and dissociation with fibrinogen and fibrin, SPR results are summarised in Table 4-1.

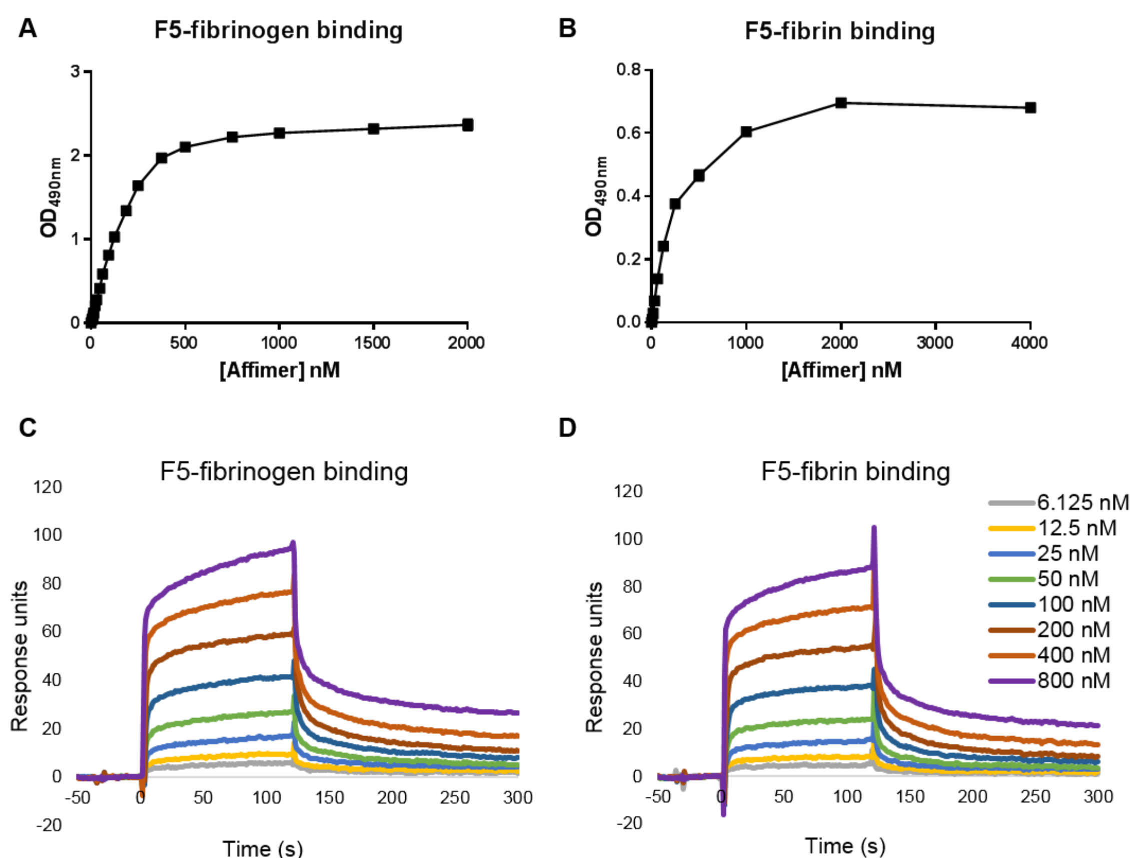


Figure 4-1 Binding characteristics of Affimer F5. The binding characteristics of Affimer F5 to fibrinogen and fibrin were assessed with plate-based ELISA assays, and SPR. **A. B.** For ELISA assays, a 96 well plate was coated with fibrinogen or fibrinogen converted to fibrin, and Affimer added in increasing concentration. After washing, Affimer was detected. Representative binding curves are shown, data presented as mean±SD of a single experiment. **C.** Binding kinetics of Affimer F5 interaction with fibrinogen and **D.** fibrin using Biacore SPR. Affimer proteins (6.125-800 nM) were injected over a fibrinogen- or fibrin- derivatised surface before following complex dissociation. n=3, representative binding data are shown. Data from Kearney et al. (2018).

	K_D ± SD (nM)	k_a ± SD (M^{-1} s^{-1})	k_d ± SD (s^{-1})
Fibrinogen	52±1.3	3.6±1.3 x10^5	0.017±0.002
Fibrin	58±9.3	4.0±0.6 x10^5	0.023±0.007

Table 4-1 Summary of binding affinities and kinetics of Affimer protein F5 studied by SPR. Affinity (K_D) and rate constants for association (k_a) and dissociation (k_d) were determined using 1:1 Langmuir binding model. n=3, mean±SD. Data from Kearney et al. (2018).

4.2.2 Affimer F5 had a dose-dependent effect on the prolongation of clot lysis in plasma and purified systems

F5 has thus far been shown to prolong clot lysis in a plasma system when used at 5:1 Affimer: fibrinogen molar ratio. To gain an insight into the effects of increasing concentrations of F5 on clot formation and lysis, turbidity and lysis assays were performed in plasma and purified systems using different concentrations of F5. Figure 4-2A and Figure 4-2B show the results of dose response curves in plasma turbidimetric assays. F5 did not significantly alter lag time or maximum OD in plasma (Figure 4-2C,D), but did prolong plasma clot lysis in a dose-dependent manner. F5 increased lysis time from 14.0±5.0 to 84.3±28.9 min at 5:1 Affimer:fibrinogen molar ratio ($P \leq 0.01$) and to 189.8±32.8 min at 10:1 ($P \leq 0.0001$) (mean±SD) (Figure 4-2E).

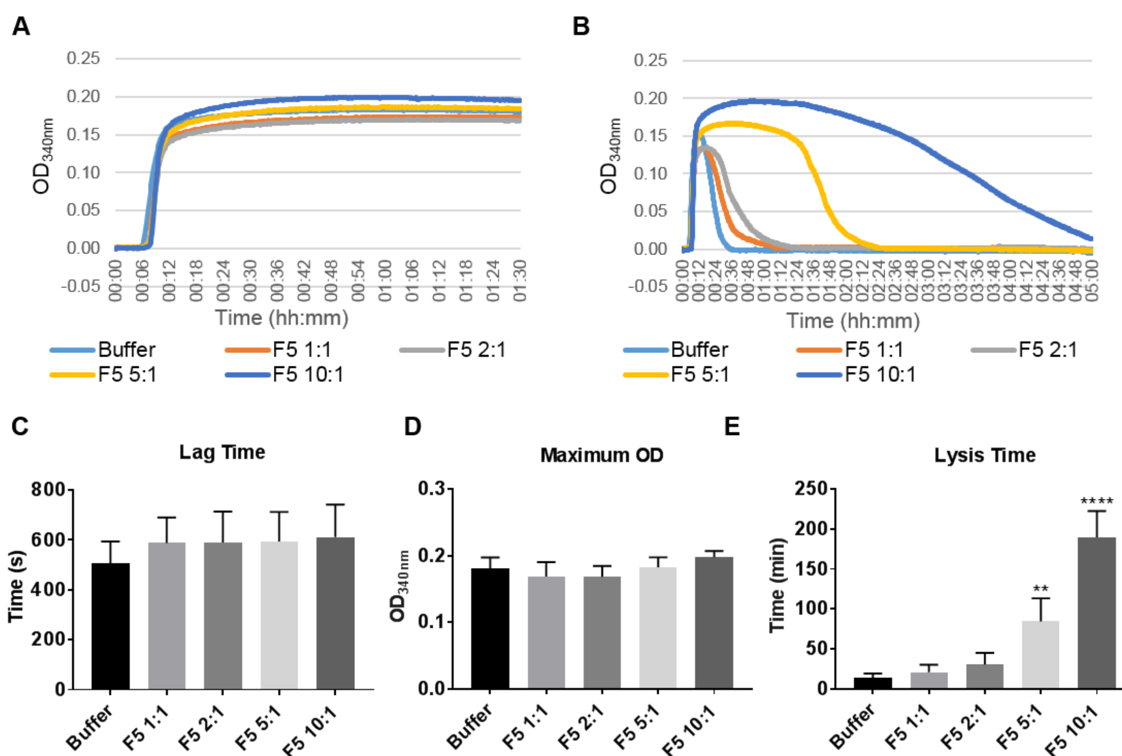


Figure 4-2 Effect of increasing doses of Affimer F5 on plasma clot formation and lysis. Pooled human plasma was pre-incubated with F5 at 1:1, 2:1, 5:1, 10:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) or thrombin, calcium and tPA (turbidity and lysis assays). Buffer only control contained no Affimer. **A.** Representative turbidity curves. **B.** Representative turbidity and lysis curves. **C.** Lag Time **D.** Maximum OD. **E.** Lysis time. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, **P≤0.01 or ****P≤0.0001 represents difference from buffer only control.

In purified turbidimetric assays (Figure 4-3A-E), F5 showed a concentration-dependent increase in lag time with more than two fold prolongation with the highest Affimer concentration (P≤0.0001) (Figure 4-3C). F5 significantly decreased maximum OD from 0.236±0.002 to 0.185±0.004 at 1:1 (P≤0.0001), 0.176±0.018 at 2:1 (P≤0.0001) and 0.196±0.001 at 5:1 (P≤0.001) (Figure 4-3D). F5 significantly increased lysis time at 5:1 and 10:1. Lysis time was prolonged from 16.5±3.5 to 37.2±9.2 min at 5:1 (P≤0.05) and to 52.0±11.9 min at 10:1 (P≤0.001) (mean±SD) (Figure 4-3E).

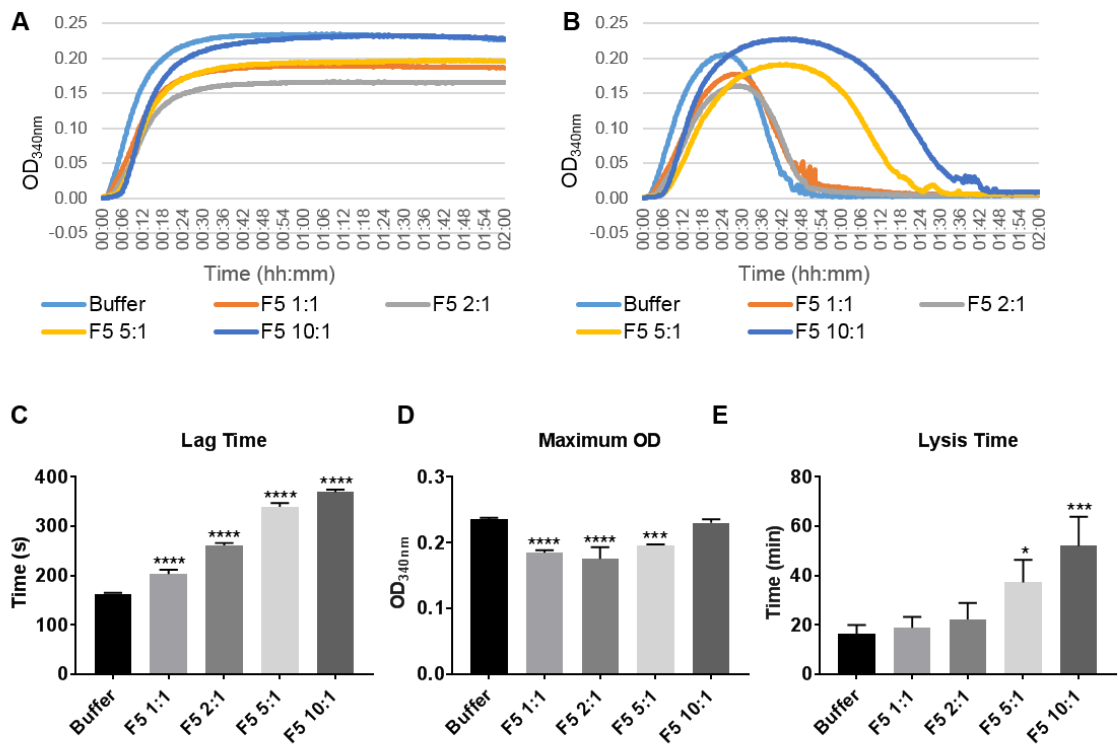


Figure 4-3 Effect of increasing doses of Affimer F5 on clot formation and lysis in a purified system. Fibrinogen was pre-incubated with F5 at 1:1, 2:1, 5:1, 10:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) or thrombin, calcium, tPA and plasminogen (turbidity and lysis assays). Buffer only control received no Affimer. **A.** Representative turbidity curves. **B.** Representative turbidity and lysis curves. **C.** Lag Time **D.** Maximum OD. **E.** Lysis time. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05, ***P≤0.001 or ****P≤0.0001 represents difference from buffer only control.

4.2.3 Prolongation of lysis by Affimer F5 is consistent in healthy individuals

To establish inter-individual variability in response, Affimer F5 was tested in individual plasma samples from healthy controls. Lag time was increased by F5 from 209±189 to 1223±279 s (P≤0.001) (mean±SD) (Figure 4-4A). F5 decreased clot maximum absorbance from 0.404±0.061 to 0.217±0.062 (P≤0.0001) (mean±SD) (Figure 4-4B) and prolonged lysis time from 7.05±1.67 to 14.87±4.74 min (P≤0.001) (mean±SD) (Figure 4-4C).

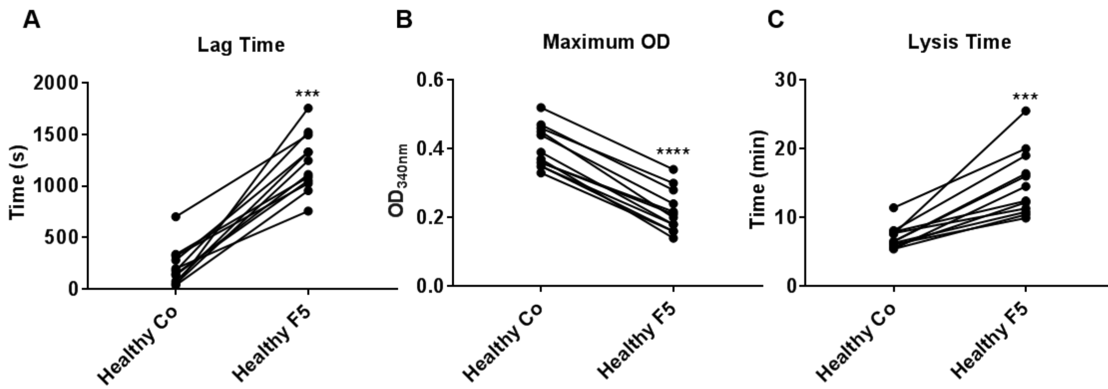


Figure 4-4 Effect of F5 on clot formation and lysis in plasma from healthy individuals. Human plasma was pre-incubated with F5 or scaffold only control protein (Co) at 5:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) and thrombin, calcium and tPA (turbidity and lysis assays). The effect of F5 on **A.** Lag time **B.** Maximum OD **C.** Lysis time. Statistical analysis was performed with a two-tailed Wilcoxon matched-pairs signed rank test (A,C) or two-tailed, paired Student *t*-test (B), *** P≤0.001 or ****P≤0.0001 represents difference from scaffold only control. Data from Kearney et al. (2018).

4.2.4 Affimer F5 prolongs lysis in plasma deficient in FVIII

Affimer F5 has thus far been shown to have an anti-fibrinolytic effect in plasma from healthy individuals. Given the potential clinical application of an anti-fibrinolytic protein in bleeding disorders, the effects of F5 on clot lysis were investigated in plasma deficient in factor FVIII (consistent with the clinical presentation of haemophilia A). Turbidimetric experiments were performed in which clotting was initiated with tissue factor, to account for the effects of FVIII deficiency, with similar experiments performed in normal pool plasma (control clots, Figure 4-5A,B). In control clots, lysis time in buffer only control was 18.37±0.9 min, and was significantly shorter in FVIII deficient plasma at 11.83±0.8 min (P≤0.001) (Figure 4-5C,D), reflecting impaired clot stability in the FVIII deficient plasma. The addition of Affimer F5 to FVIII deficient plasma prolonged lysis from 11.83±0.8 min to 23.77±1.2 (P≤0.05) and 36.77±8.2 (P≤0.001) min at 5:1 and 10:1 Affimer:fibrinogen molar ratios thus normalising, and even prolonging, the short lysis time in this condition (Figure 4-5C,D).

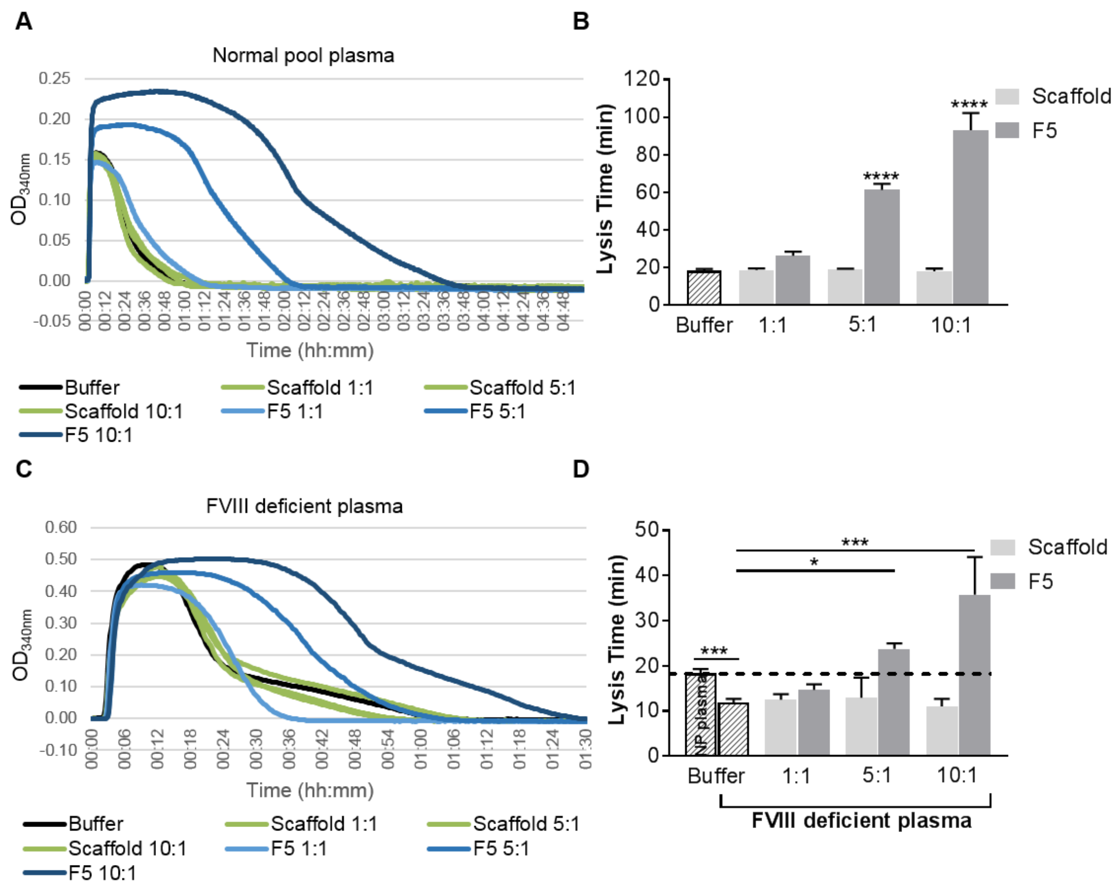


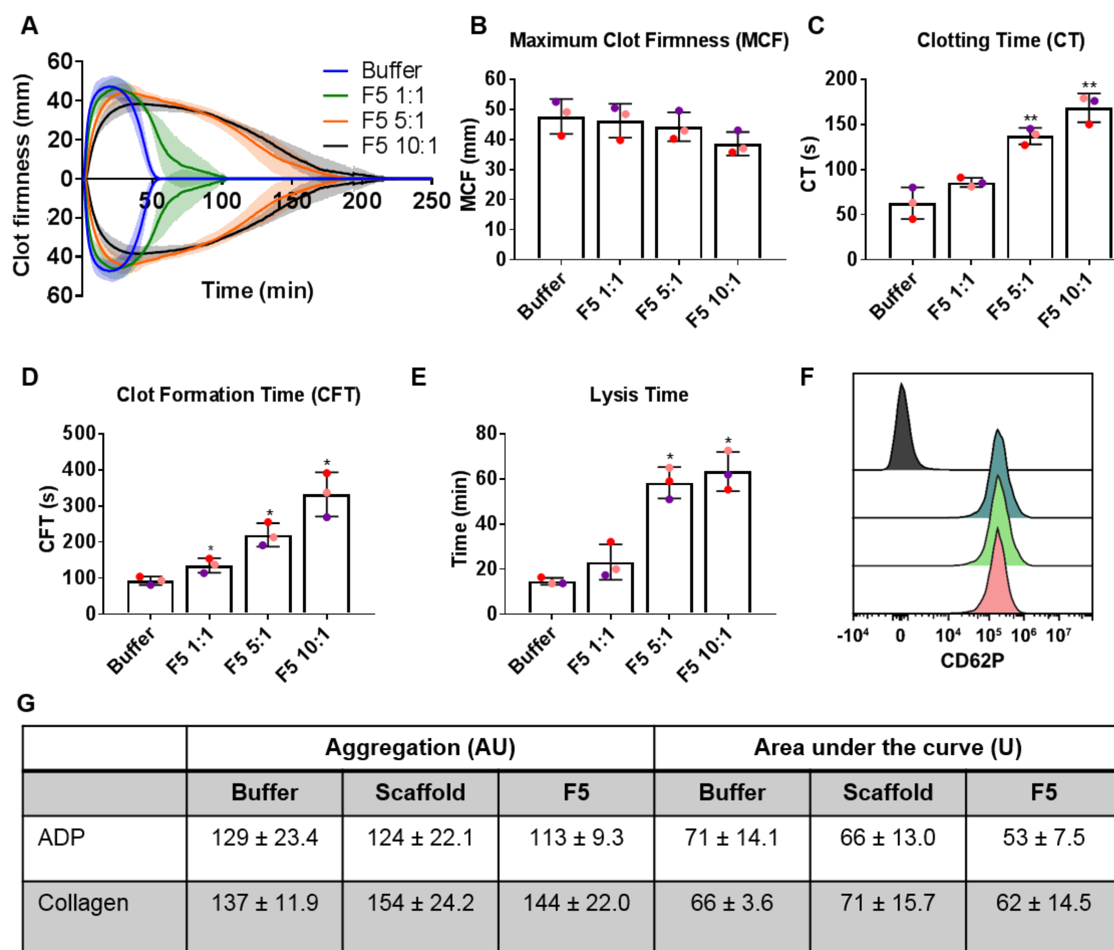
Figure 4-5 Effect of F5 on clot lysis in normal pool plasma and FVIII deficient plasma. Turbidimetric experiments in which clot formation was initiated with tissue factor (5 pM) were performed using **A.B.** normal pool (NP) plasma and **C.D.** FVIII deficient plasma. The effect of increasing concentrations of Affimer F5 were tested, with Affimer scaffold protein included as a control. **A.C.** show representative turbidity and lysis curves. **B.D.** numbers on the x-axis represent Affimer protein:fibrinogen molar ratio. n=3, mean±SD. One-way ANOVA was used to determine the significance of Affimer F5 effect at different Affimer concentrations, *P≤0.05; ***P≤0.001, ****P≤0.0001 represent difference from buffer only control. Unpaired Student *t*-test was used to compare buffer only controls in normal and FVIII deficient plasma, ***P≤0.001. Data from Kearney et al. (2018).

4.2.5 The effect of Affimer protein F5 on platelet function and fibrinolysis in whole blood

F5 has been shown to prolong lysis in both a purified and plasma based system. To ensure that cellular elements did not compromise the anti-fibrinolytic properties of the Affimer, ROTEM with tPA was used to investigate the effect of increasing concentrations of the Affimer on clot formation and lysis in whole blood from healthy individuals. Figure 4-6A shows that Affimer F5 caused a significant concentration-dependent prolongation of lysis time. Lysis time was prolonged from 14.6±1.6 min in the absence of Affimer to 58.3±7.0 and 63.3±8.7 min (P≤0.05) at 5:1 and 10:1 Affimer:fibrinogen molar ratio, respectively (Figure

4-6E), suggesting that molar ratios above 5:1 have little additional effect. The presence of the Affimer did not significantly affect maximum clot firmness (Figure 4-6B), but did prolong clotting time from 62.7±17.5 s in buffer control to 137.0±9.2 and 168.3±16.0 s at 5:1 and 10:1 Affimer:fibrinogen molar ratio, respectively ($P \leq 0.01$) (Figure 4-6C). Affimer F5 also prolonged clot formation time at 1:1, 5:1 and 10:1, from 92.7±11.5 s in buffer control to 135.0±20.1, 219.7±32.5 and 332.3±61.1 s, respectively ($P \leq 0.05$) (Figure 4-6D).

The effect of the Affimer on platelets in whole blood was investigated using flow cytometry performed by Dr Benjamin Spurgeon. In TRAP stimulated blood samples, platelet expression of CD62P was not affected by Affimer F5 (Figure 4-6E). The Affimer also had no effect on ADP- or collagen-induced platelet aggregation in whole blood (Figure 4-6F) when analysed using the Multiplate Analyser by Fladia Hawkins.



	Aggregation (AU)			Area under the curve (U)		
	Buffer	Scaffold	F5	Buffer	Scaffold	F5
ADP	129 ± 23.4	124 ± 22.1	113 ± 9.3	71 ± 14.1	66 ± 13.0	53 ± 7.5
Collagen	137 ± 11.9	154 ± 24.2	144 ± 22.0	66 ± 3.6	71 ± 15.7	62 ± 14.5

Figure 4-6 Effect of Affimer F5 on lysis and platelet activation in whole blood. A. Whole blood was added to ROTEM cups with ex-tem and star-tem reagent, with Affimer to final concentrations of 1:1, 5:1, 10:1 Affimer:fibrinogen molar ratio. Buffer only control received no Affimer. tPA was also added to the reaction mixture to allow clot lysis. ROTEM traces from three healthy volunteers, mean±SD. **B.** ROTEM-calculated parameter maximum clot firmness (MCF). **C.** ROTEM-calculated parameter clotting time (CT). **D.** ROTEM-calculated parameter clot formation time (CFT). **E.** Time from MCF to 50% clot lysis. Data in B, C, D and E presented as mean±SD. Each individual's data is presented in a different colour (pink, red and purple) to show inter-individual variability. Statistical analysis was performed using repeated measures one-way ANOVA, *P≤0.05, **P≤0.01 when compared with buffer only control. **E.** The effect of F5 on platelet activation was tested using whole blood from three healthy volunteers. Blood was incubated with buffer only (dark green peak), scaffold (light green), or F5 (pink) and then stimulated with TRAP. CD62P expression was compared with basal platelets (grey peak). Affimer proteins were used at 10:1 Affimer:fibrinogen molar ratio. **F.** Whole blood aggregation was assessed in the presence of Affimer F5 and scaffold control protein (at 10:1 Affimer:fibrinogen molar ratio) using ADP and collagen as agonists and was quantified as aggregation (expressed in aggregation units AU) and area under the curve (expressed in arbitrary units, AU*min or U; 1U=10AU*min). n=3, mean±SD. Statistical analysis was performed using one-way ANOVA comparing with buffer only. Data from Kearney et al. (2018).

4.2.6 F5 causes formation of a more porous clot with thicker fibres

Confocal microscopy analysis of clots formed in the presence of F5 allowed the 3D structure to be viewed in hydrated conditions, and showed that, while preserving physiological structure, the Affimer caused formation of clots with fewer fibres (Figure 4-7A,C). The reduction in fibre count was small but statistically significant, clots formed without Affimer had an average of 35.5 fibres per 100 μm, which was decreased in the presence of Affimer F5 to 32.1 ($P \le 0.01$) and 28.2 ($P \le 0.0001$) fibres per 100 μm at 5:1 and 10:1 Affimer:fibrinogen molar ratio, respectively (Figure 4-7C). For a closer look at the effect of F5 on fibrin fibre structure, clots made with the Affimer were viewed by SEM (Figure 4-7A). Clots formed in the presence of F5 appeared less dense and had significantly thicker fibres, fibre thickness was increased in a concentration-dependent manner (Figure 4-7D). Control clots made in the presence of scaffold protein did not significantly differ from clots made with buffer only (Figure 4-7B,C,D).

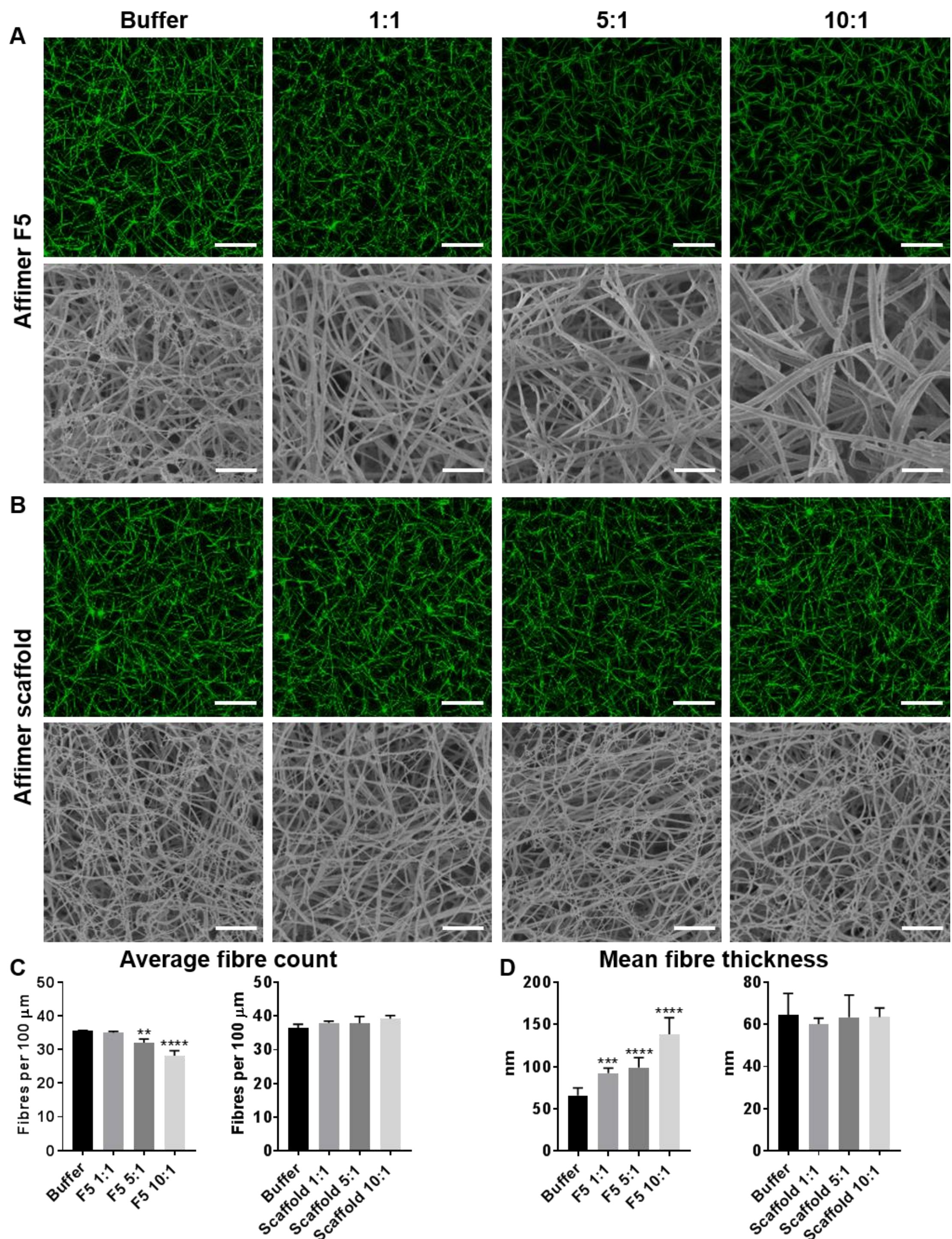


Figure 4-7 Confocal and SEM images of plasma clots in the presence of Affimer F5 and Affimer scaffold. Affimers were used in plasma at 1:1, 5:1 and 10:1 Affimer:fibrinogen molar ratio. Confocal images (top) and SEM images (bottom) of **A.** clots with Affimer F5 and **B.** Affimer scaffold. Scale bar on confocal images = 20 μm, on SEM images = 1 μm. **C.** Average fibre count in confocal images of clots. Two clots were made for each condition, and 3 images taken in different areas of each clot. Fibre density was determined by counting the number of fibres that cross 20 lines across each image, using ImageJ software. **D.** Mean fibre thickness (nm) of fibres in SEM images. Two clots were made for each condition and images taken in 5 different areas of each clot. 15 fibres were measured in each image at 10K magnification in ImageJ software. Data presented as mean±SD. Statistical analysis was performed using one-way ANOVA, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ represents difference from buffer only control. Data from Kearney et al. (2018).

SEM and confocal data were consistent with permeation experiments, in which plasma clots were formed in the presence of F5 or control Affimer scaffold and the movement of assay buffer through the clot measured over time. Permeation experiments were performed by Dr Ramsah Cheah. The Darcy constant, which describes the movement of fluid through a porous medium, was significantly increased for clots formed with F5, suggesting a more porous clot. Clots formed with scaffold did not have significantly altered permeation properties (Table 4-2).

Sample	n	K_s ± SD (10^{-9} cm^2)
Buffer	8	5.01 ± 0.77
F5 5:1	7	9.05 ± 2.00**
F5 10:1	7	12.99 ± 4.32****
Scaffold 5:1	5	3.94 ± 1.38
Scaffold 10:1	8	6.07 ± 1.71

Table 4-2 The permeation properties of plasma clots, formed with and without Affimer proteins. Clots were made in the presence of buffer only, Affimer F5 and scaffold control protein at 5:1 and 10:1 Affimer:fibrinogen molar ratio in pooled human plasma. The Darcy constant (Ks) of each sample was calculated using the flow measurements acquired. Data presented as mean±SD, **P≤0.01, ****P≤0.0001 when compared with buffer only control based on one-way ANOVA. Data from Kearney et al. (2018).

4.2.7 Affimer F5 binds fibrinogen in solution

To confirm fibrinogen specificity of Affimer F5 in plasma, a pull down assay was developed. Two types of magnetic beads with affinity for histidine (His)-tagged proteins were tested with and without F5 in plasma to determine their suitability for Affimer pulldowns (Figure 4-8A,B). Both HisPur and Dynabeads were tested with a range of salt concentrations within the limits recommended by the manufacturer to identify conditions that reduced non-specific binding to the affinity beads (Figure 4-8A,B). Dynabeads were chosen for their reduced non-specific binding of protein at the highest concentration of salt used in the pull down wash buffers (3 µg non-specific protein eluted from Dynabeads vs. 8.8 µg eluted from HisPur beads).

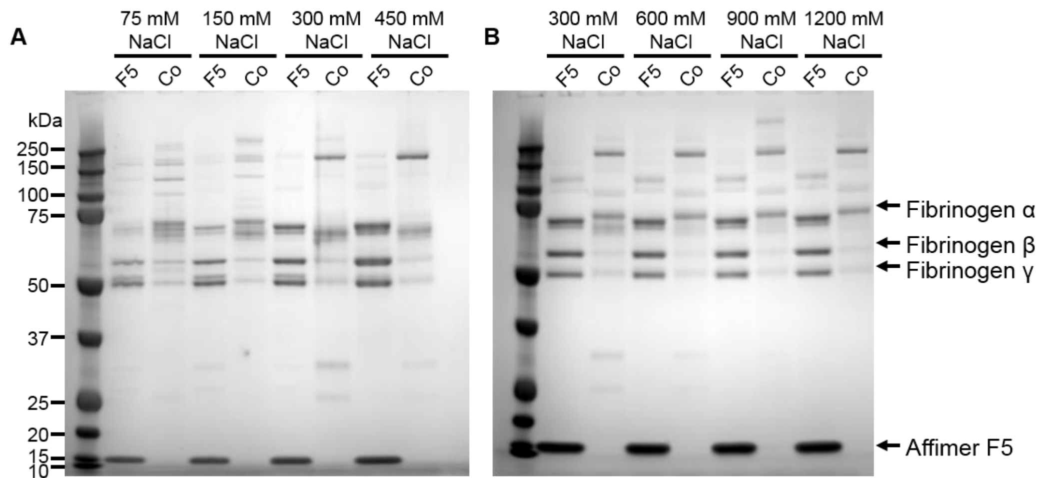


Figure 4-8 Pull down assay method development. A pull down assay protocol was developed to determine the fibrinogen-specificity of F5 in plasma. Two types of his-tag binding beads were used in a pull down assay (HisPur and Dynabeads). Each set of beads was tested using Affimer F5 and a bead-only control (Co). Protein eluted from the bead-only control was protein binding non-specifically to the bead. **A.** Elutions from pull downs using HisPur beads with increasing [NaCl] in the wash buffers to reduce background binding to the beads. The concentration of NaCl in the wash buffers is shown at the top of each lane. **B.** Dynabead pull downs with increasing [NaCl] in the wash buffers. The concentration of NaCl in the wash buffers is shown at the top of each lane. The location of Affimer F5 (~13 kDa) and fibrinogen chains are indicated.

Figure 4-9A shows pulldown elutions of Affimers F5, anti-SUMO and scaffold in plasma, compared with elutions from bead-only control (no Affimer). F5 was able to pull fibrinogen out of pooled human plasma (confirmed with anti-fibrinogen western blot in Figure 4-9B). Binding of fibrinogen to Affimer F5 may have prevented other protein-Affimer interactions from occurring by steric hindrance, and so a pulldown was performed in pooled plasma from afibrinogenemic (a-fib) patients and compared with normal pool (NP) plasma, to investigate protein-Affimer interactions in the absence of fibrinogen (Figure 4-9C). Again, F5 was able to pull fibrinogen out of NP plasma, and a small amount of fibrinogen from a-fib patient plasma (Figure 4-9C). Two bands, one at ~110 kDa and one at ~90 kDa were identified in Affimer pulldowns, and in the bead-only controls (although the bands appear enriched in the Affimer samples) (Figure 4-9C,D). These proteins were thought to be potential binders to the Affimer scaffold and were subsequently identified by mass spectrometry. Band 1 contained primarily kininogen-1 (at expected MW 110 kDa) and band 2 contained primarily fibrinogen γ chain (at MW of ~90 kDa, consistent with γ-γ dimers) (Figure 4-9D).

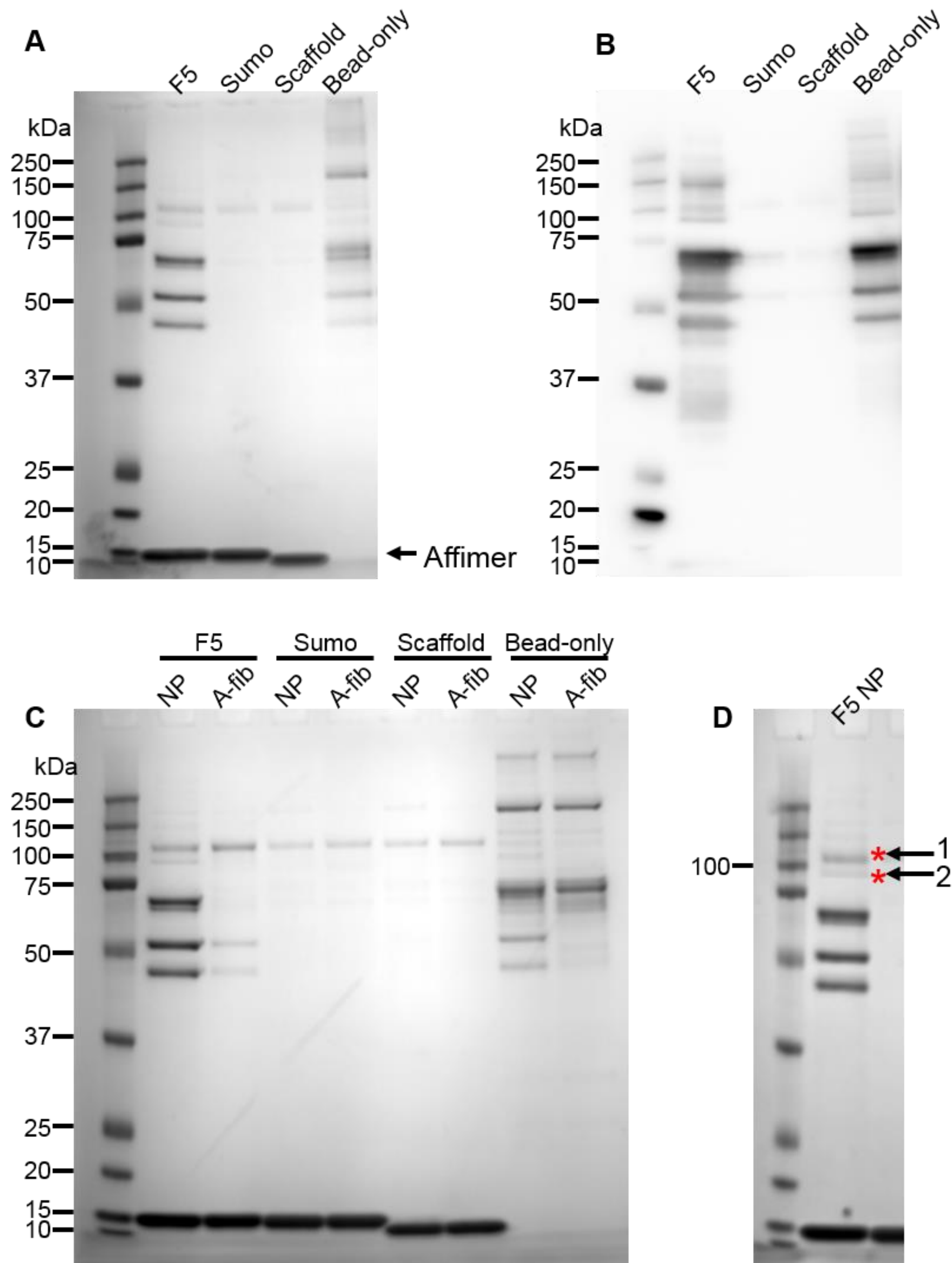


Figure 4-9 Pull down assays with F5, anti-SUMO (sumo) and scaffold in plasma to determine the fibrinogen specificity of Affimers. F5, sumo and scaffold were incubated with Dynabeads to allow the C-terminal his-tag of the Affimers to adhere to the Dynabead surface. A bead-only control was also included, which was incubated with assay buffer only (no Affimer protein). Dynabeads were then incubated with plasma for 30 min prior to washing, and elution of proteins from the Dynabeads. **A.** Pull down elutions run on a reducing gel. **B.** Anti-fibrinogen blot of pull down elutions using a polyclonal anti-fibrinogen antibody. **C.** Pull down assay with F5, sumo and scaffold in normal pool human plasma (NP) and pooled plasma from afibrinogenemic patients (A-fib). **D.** F5 pull down elutions were re-run on a gel, and the bands indicated by * were identified by mass spectrometry. Band 1 contained primarily kininogen-1 and band 2 contained primarily fibrinogen γ chain.

4.2.8 Conformational and not linear interactions are responsible for changes in clot structure/lysis

Linear peptides of the same sequence as the two variable regions of Affimer F5 were synthesized and their effect on clot formation and lysis was tested using the turbidity and lysis assay in pooled human plasma. The synthetic peptides had no significant effect on lysis time when used either on their own (Figure 4-10A,B) or in combination (Figure 4-10C). These data suggested that the interaction between the Affimer and fibrinogen was dependent on the constraint of the Affimer variable regions within the Affimer scaffold. Molecular modelling simulations of Affimer F5 (Figure 4-10D), performed by Dr Katie Simmons, demonstrated that the variable region loops were held in compact conformations when within the Affimer scaffold (Figure 4-10E) but were linear and highly flexible when modelled as isolated 9 amino acid peptides (Figure 4-10F).

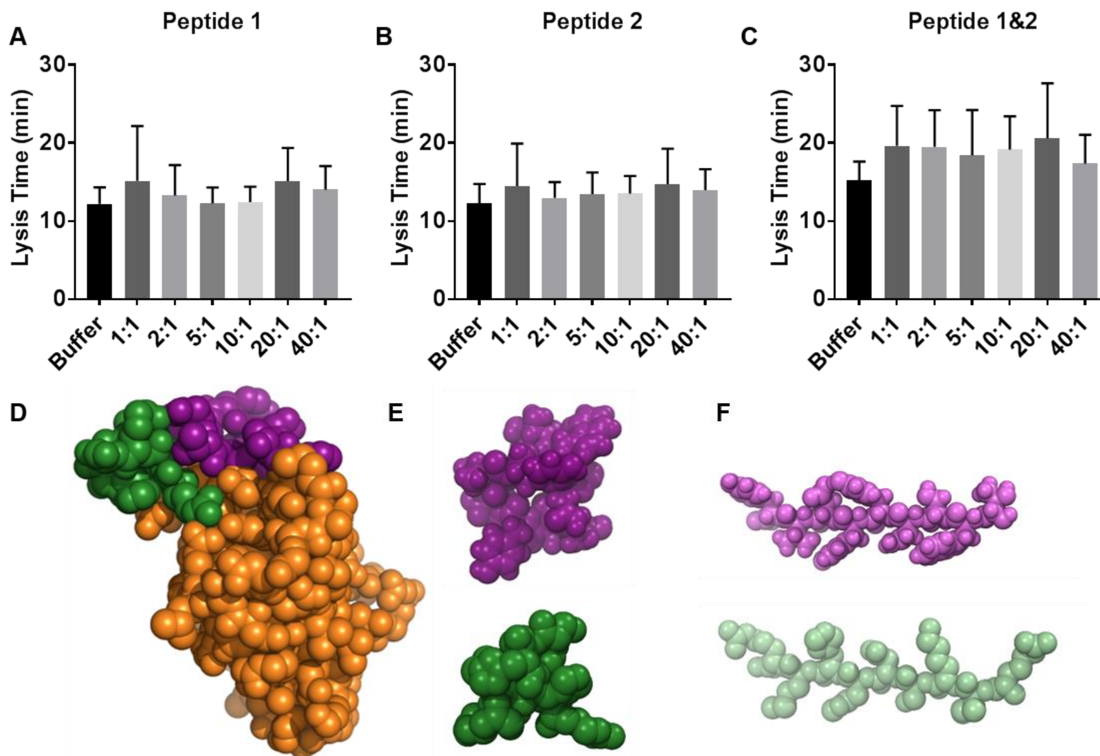


Figure 4-10 The effect of Affimer variable region synthetic peptides on clot lysis in plasma. Synthetic peptides with the amino acid sequence of Affimer F5's two variable regions were produced, and added to pooled human plasma in a turbidity and lysis assay to assess their effect on clot lysis. Peptides were used at 1:1, 2:1, 5:1, 10:1, 20:1 and 40:1 peptide:fibrinogen molar ratio. Buffer only control received no peptide protein. Lysis time in assays using **A.** Peptide 1 **B.** Peptide 2 **C.** Peptide 1 and 2 together. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, comparing buffer only control with each peptide:fibrinogen molar ratio used. **D.** Model of Affimer F5, based on the crystal structure of the Affimer scaffold (PDB ID 4N6T). The Affimer scaffold is orange, variable region one is purple, and variable region two is green. **E.** Variable region one (purple) and two (green) in the conformation these regions adopt when part of the Affimer scaffold. **F.** The sequences of the Affimer's variable regions were modelled as two 9 amino acid peptides using the Maestro graphical user interface, and energy minimised using the OPLS3 force-field in a water environment.

4.2.9 F5 is incorporated into the clot, and prolongs lysis by a mechanism independent of changes to clot structure

To begin to investigate the mode of action of F5, the Affimer was labelled using Alexa-488 dye (green), and incorporation into plasma clots made in the presence of Alexa-594 labelled fibrinogen (red) was assessed. Figure 4-11A shows that F5 was incorporated into the clot, as signal from the green (Affimer) channel colocalised with the red (fibrinogen) signal. As a control, scaffold protein was fluorescently labelled, and showed no incorporation into the clot (Figure 4-11A).

Thus far, the effect of Affimer F5 on prolongation of lysis has been investigated in assays in which the Affimer is added from the start – and so is present during clot formation and able to influence fibrin clot structure. However it was also of interest to investigate the ability of Affimer F5 to bind to already-formed clots and exert an anti-fibrinolytic effect without altering clot structure. Clots were made with pooled human plasma in a turbidity assay, and Affimer added to the clots once they were fully formed immediately before the addition of lysis mix. F5 showed an ability to limit clot lysis under these conditions. After 300 min of lysis, buffer only control had reached 100% lysis, which was significantly reduced in F5 samples to 53.7±7.0 % lysis (mean±SD) ($P \leq 0.001$) (Figure 4-11B). Control Affimers scaffold and anti-SUMO did not delay clot lysis in similarly performed experiments (Figure 4-11C). These data confirm a specific ability of Affimer F5 to exert a fibrin-mediated mechanism of prolongation of clot lysis.

These data show that although F5 prolongs lysis when present during clot formation (and can be incorporated into the fibrin clot), the Affimer also functions when added to a fully formed clot, through mechanisms independent of Affimer-induced changes to fibrin structure.

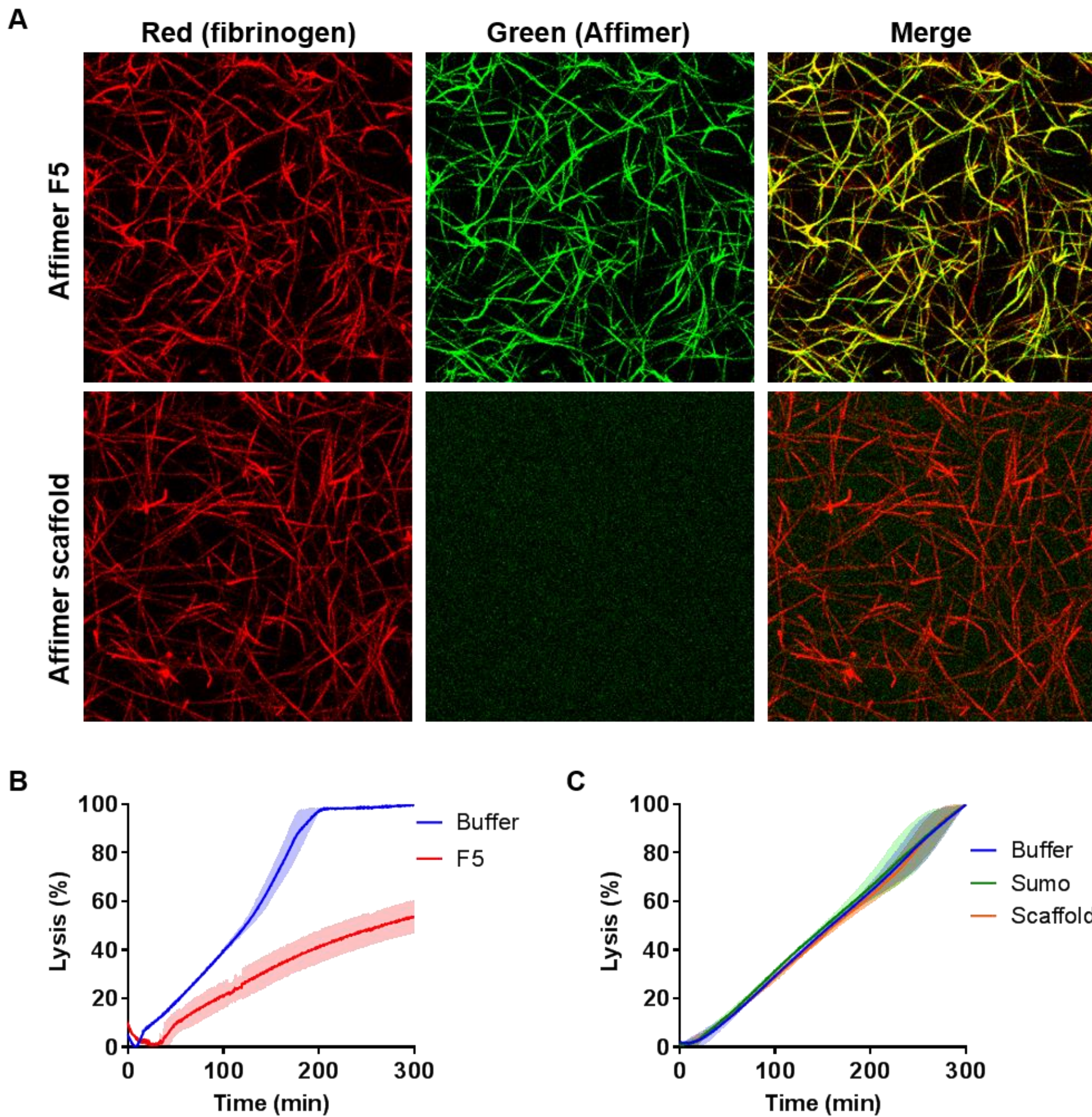


Figure 4-11 Incorporation of F5 into the fibrin clot, and lysis of pre-formed plasma clots in the presence and absence of Affimer. A. Confocal images of plasma clots pre-incubated with either Alexa-488 labelled F5 or scaffold and Alexa-594 labelled fibrinogen. Affimers were added to clots at 5:1 Affimer:fibrinogen molar ratio. Fibrinogen appears red, Affimers green and merged red-green signal appears yellow. **B.** Turbidity and lysis assays were performed in which the Affimer was not present during clot formation. Pooled human plasma was clotted with thrombin and calcium, and left for 1 h to reach maximum turbidity. After 1 h, Affimer F5 (at 5:1 Affimer:fibrinogen molar ratio) or buffer was added to the clot immediately before the addition of tPA. **B.** shows clot lysis (%) over time of clots which received Affimer F5 (red lines) or buffer (blue lines) before lysis mix. **C.** A similarly performed control experiment with anti-SUMO (sumo, green) and scaffold (orange) proteins. n=3, mean±SD.

4.2.10 Fibrin degradation in the presence of Affimer F5

Given the ability of F5 to prolong lysis through mechanisms independent of changes to fibrin clot structure, it was thought that F5 may be able to limit fibrin degradation by a more direct effect on plasmin interaction with fibrin. To investigate the fibrin degradation products (FDPs) formed in the presence of Affimer F5, clots were made with and without the Affimer in Calbiochem fibrinogen (which contains FXIII) [335] and IF-1 purified fibrinogen (largely depleted of FXIII [335]). Clot lysis was stopped at different time points, and the lysis products run on reducing gels. Figure 4-12 shows that different FDPs were formed in the presence of the Affimer in both IF-1 purified and Calbiochem fibrinogen. The identity of FDPs could be determined by comparison with those identified by Walker and Nesheim (1999) [102] and Pizzo et al. (1973) [336], who performed similar experiments. In Figure 4-12B, there are reduced quantities of γ dimer species in the presence of F5 when compared to clots formed without F5 in Calbiochem fibrinogen (Figure 4-12A), reflecting a delay in lysis of crosslinked fibrin in the presence of the Affimer. There are bands corresponding to uncrosslinked γ chains in clots formed with F5 (Figure 4-12B), but not in those formed without the Affimer (Figure 4-12A). Additionally, there is a band corresponding to lysis of uncrosslinked γ chain (γ') in clots formed with F5 (Figure 4-12B) and not in those formed without F5 (Figure 4-12A). In clots formed in IF-1 purified fibrinogen, which is relatively free of FXIII, there is a small quantity of crosslinked γ species in clots formed without F5 (Figure 4-12C), which are largely missing in the presence of F5 (Figure 4-12D).

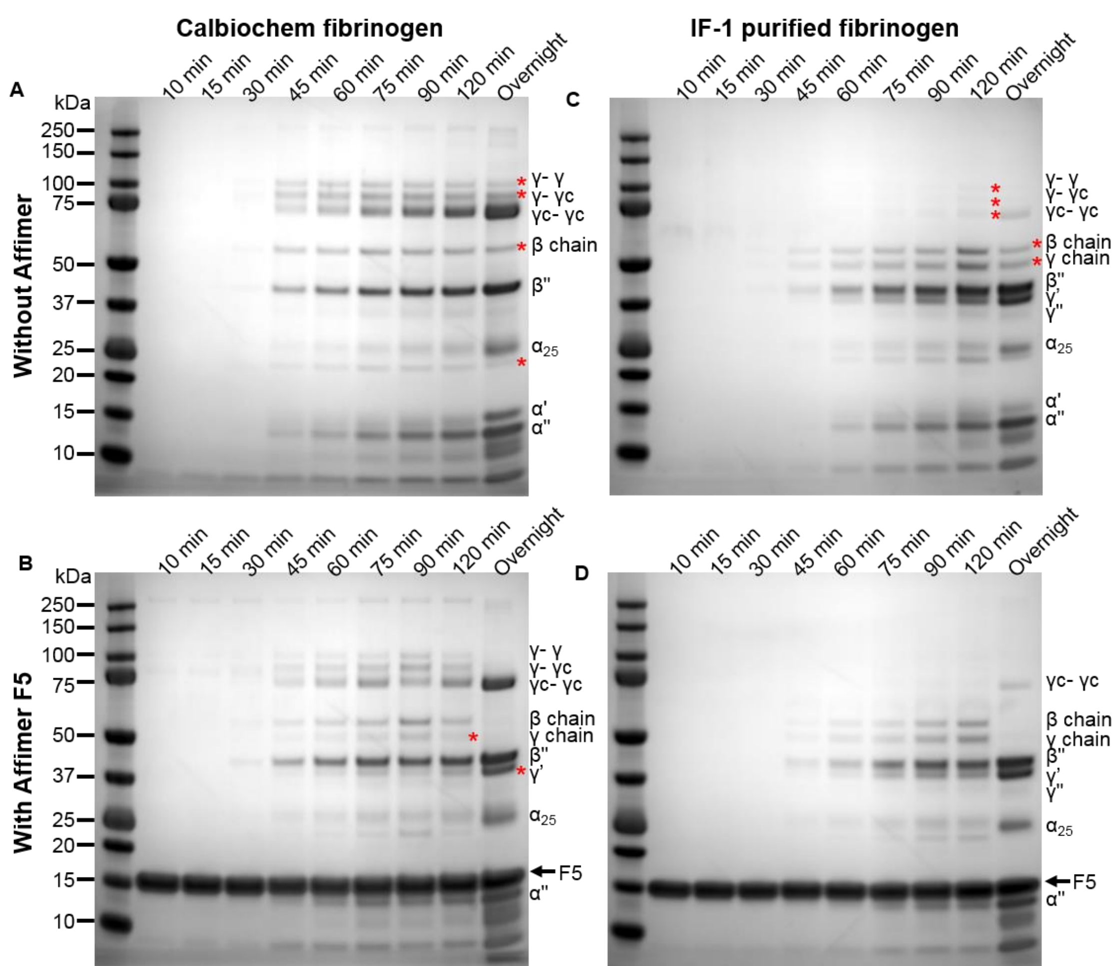


Figure 4-12 Fibrin degradation products of clots formed in the presence and absence of Affimer F5. Clots made from either Calbiochem fibrinogen or IF-1 purified fibrinogen were formed with and without Affimer F5, and incubated overnight at 37 °C. The next day, lysis mix containing tPA and plasminogen was added to all clots. Lysis was stopped by the addition of plasmin inhibitor aprotinin at different time points (indicated above gels). The clots were spun down and the supernatant analysed by SDS-PAGE. **A. B.** Lysis products of clots formed with Calbiochem fibrinogen **A.** without Affimer F5 and **B.** with Affimer F5. **C.D.** Lysis products of clots formed with IF-1 purified fibrinogen **C.** without F5 and **D.** with F5. * indicates the presence of bands on the gel not present in corresponding ± F5 gel. The position of F5 is indicated with an arrow on the appropriate gels. The identity of some of the fibrin fragments, based on the known molecular weights of fibrin digestion products is indicated next to the appropriate gel bands, including γ dimers (γ-γ), γ dimers with one chain cleaved (γ-γc), γ dimers with both chains cleaved (γc-γc), partially digested γ chain (γ' or γ"), β chain, cleaved β chain (β"), α chain fragment of 25 kDa (α_{25}) or cleaved α chain (α" or α').

4.2.11 Affimer F5 interacts with the fibrinogen D region

To investigate F5-fibrinogen interaction sites, Affimer protein was incubated with fibrinogen prior to digestion with plasmin. Pull down assays were performed to isolate the Affimer and any interacting fibrinogen degradation products. Figure 4-13A shows that F5 was able to pull out a fibrinogen degradation product, that when analysed by SDS-PAGE contained protein fragments at ~40 kDa, 38 kDa, and a faint band at ~12 kDa, the latter running close to the Affimer protein band. It was suspected that these bands corresponded to the D fragment, a terminal digestion product of fibrinogen [337] that consists of the C terminal regions of the β chain (42 kDa), γ chain (38 kDa) and α chain (12 kDa) [98, 337, 338]. The content of the protein bands indicated in Figure 4-13A was confirmed by mass spectrometry, identifying the D fragment as an F5 binding partner. Additional pull down assays in which fibrinogen was lysed prior to the addition of the Affimer allowed fibrinogen digestion to proceed without potential interference by the Affimer. Figure 4-13B shows that F5 pulled down similar fibrinogen degradation products under these conditions. To confirm that the Affimer was pulling down the fibrinogen D region, a single protein fragment comprised of α, β and γ portions, pull down elutions were also run under non-reducing conditions. Under non-reducing conditions, the Affimer-interacting fibrinogen degradation product ran as a single band, consistent with the molecular weight of the fibrinogen D fragment (~90 kDa) and consistent with the sum of protein bands under reducing conditions (42 kDa β, 38 kDa γ, 12 kDa α) (Figure 4-13C).

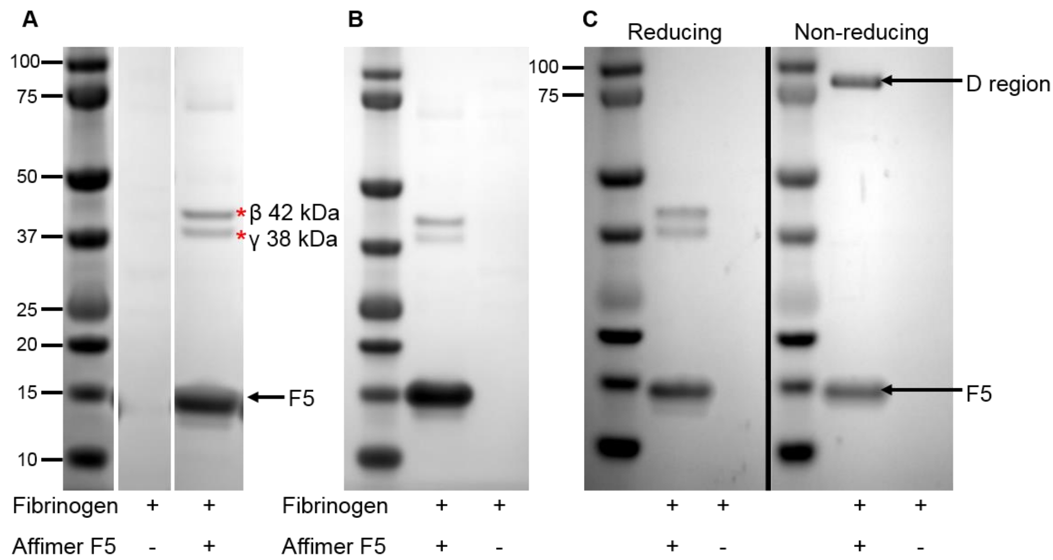


Figure 4-13 Affimer F5 interacts with the fibrinogen D region. To identify F5-fibrinogen interaction sites, a pull down was performed in which fibrinogen was digested with plasmin, before or after incubation with Affimer F5. Affimer-interacting protein complexes were isolated with affinity beads. **A.** Fibrinogen was incubated with Affimer prior to plasmin digestion for 2 h. The proteins marked with *, pulled down by the Affimer, were identified by mass spectrometry. The location of Affimer F5 is indicated with an arrow. **B.** A pull down was also performed in which fibrinogen was digested with plasmin for 2 h, prior to incubation of fibrinogen degradation products with Affimer F5, and pulldown with Dynabeads. Proteins eluted from the Dynabeads were analysed by SDS-PAGE under reducing conditions. **C.** Experiments in which fibrinogen was incubated with Affimer F5 prior to plasmin digest and pull-down were repeated, and elutions run under reducing (left) and non-reducing (right) conditions to confirm that the Affimer was interacting with a single fibrinogen fragment. Data from Kearney et al. (2018).

4.2.12 Binding of Affimer F5 to fibrinogen and fibrin

Pull down experiments with F5 and fibrinogen degradation products identified fragment D as an F5 binding partner. To further investigate this interaction, molecular modelling was performed by Dr Katie Simmons using the published crystal structures of fragment D [328] and a homology model of Affimer F5 based on the crystal structure of the Affimer scaffold [289]. Using an unbiased docking approach, Autodock 4.2 predicted binding of Affimer F5 to the γ chain of fibrinogen D fragment, in an area spatially close to the γ312-324 tPA and the α148-160 tPA/plasminogen binding site [87, 91, 92]. The region bound by F5 was occupied by both the lowest energy and most populated clusters of predicted docking poses (Figure 4-14A,B). The fibrinogen residues within 4 Å (the accepted limit for hydrogen bonding interactions) of the F5 variable region loops were similar in both docking models (Figure 4-14C).

Previous turbidimetric experiments showed an ability of Affimer F5 to exert an anti-fibrinolytic effect when added to a fully formed clot (Figure 4-11), suggesting an ability to bind to fibrin fibres, in addition to its ability to bind fibrinogen. Furthermore, ELISA and SPR studies demonstrated that the Affimer had similar affinity for fibrin as for fibrinogen (Figure 4-1). To support this concept, it was determined that the residues predicted to interact with the Affimer in the fibrinogen fragment D model remain exposed/accessible upon conversion to fibrin in the crystal structure of fibrin DD fragment (Figure 4-14D).

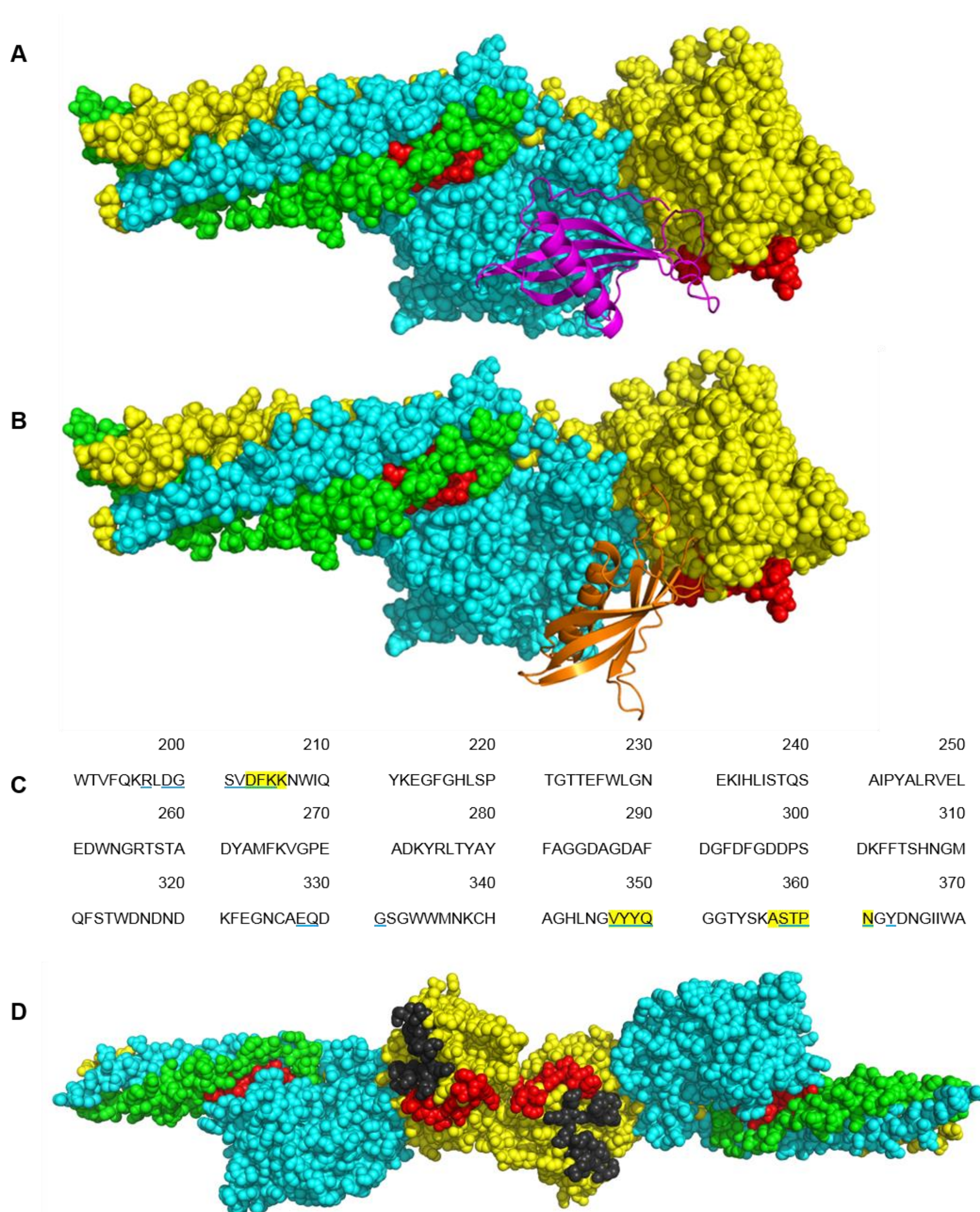


Figure 4-14 Binding of Affimer F5 to the fibrinogen D region. Autodock 4.2 prediction of Affimer F5 binding to the γ chain of the D fragment of fibrinogen (PDB ID 1FZA), in both the lowest energy and most populated poses. Fibrinogen fragment D is shown as a space-filling model, with the α chain in green, β chain in turquoise and γ chain in yellow. The tPA/plasminogen binding site α148-160 and the tPA binding site γ312-324 are highlighted in red. **A.** In the lowest energy pose, the variable region loops of F5 (depicted with magenta ribbons) are interacting with the fibrinogen γ chain near to the tPA binding site. **B.** The most populated clusters of predicted docking poses also placed Affimer F5 (orange ribbons) in a similar area of fibrinogen fragment D. **C.** Amino acid sequence of fibrinogen γ chain, with those amino acids within 4 Å of Affimer F5's variable region loops marked. Fibrinogen residues close to Affimer loops in the lowest energy pose are highlighted yellow and in the most populated pose are underlined blue. Numbering of amino acid residues is based on full length sequence of fibrinogen γ chain (Uniprot ID 20679). **D.** Fibrin DD crystal structure (PDB ID 1FZC). Residues predicted to interact with F5 (underlined/highlighted in (C)) are coloured black, to show their exposed location in this structure. Data from Kearney et al. (2018).

4.2.13 Mechanisms for the anti-fibrinolytic effects of Affimer F5

Given the binding of Affimer F5 to the fibrinogen D region, and the predicted site of interaction in close proximity to both the α148-160 tPA/plasminogen and the γ312-324 tPA binding site, plasminogen to plasmin conversion in the presence of Affimer was assayed using chromogenic substrate S2251. When Affimer F5 was present during clot formation, the rate of plasmin generation was reduced at all Affimer concentrations used from 0.062±0.003 au/min in buffer only controls to 0.052±0.001 ($P \leq 0.001$), 0.041±0.001 ($P \leq 0.0001$), 0.036±0.001 ($P \leq 0.0001$) au/min at 1:1, 5:1, 10:1 Affimer:fibrinogen molar ratios respectively, while scaffold and anti-SUMO control proteins had no effect (Figure 4-15A,B). The same assay was also performed without fibrinogen, in the presence and absence of Affimer F5 to assess the fibrinogen specificity of the Affimer-induced reduction in plasmin generation rate. There was no significant difference in plasmin generation rate, suggesting the effects of the Affimer were fibrin(ogen)-specific (Figure 4-15C).

To further test the fibrinogen specificity of the Affimer, turbidity and lysis assays were performed using plasma samples in which clots were formed with and without Affimer F5, and lysed with urokinase type plasminogen activator (uPA). These assays showed no significant effect of the Affimer on uPA-induced lysis Figure 4-15D, in agreement with the importance of F5 interaction with the fibrin network to exert its anti-fibrinolytic effect (since uPA-induced plasminogen conversion to plasmin is not fibrin-dependent [339]).

To investigate the mechanism of reduced rate of S2251 hydrolysis, a turbidity and lysis assay using purified fibrinogen was performed. Clots formed in the presence of F5 protein showed delayed lysis when compared with controls in samples lysed with plasminogen/tPA, but not plasmin (Figure 4-15E). These data indicate an effect of F5 on plasminogen to plasmin conversion, and not directly on the plasmin lysis of clots.

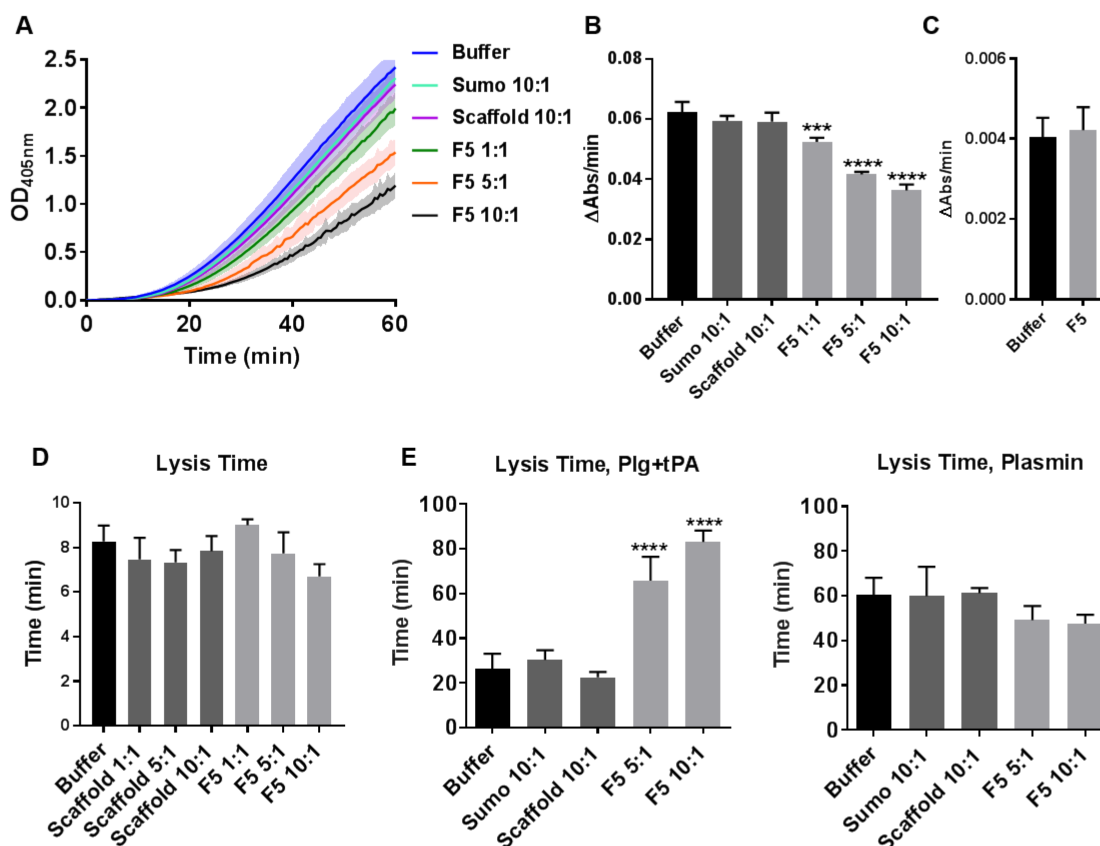


Figure 4-15 Affimer F5 reduced the rate of plasminogen to plasmin conversion in a fibrin(ogen) specific manner. A. Plasminogen conversion to plasmin was assessed by monitoring plasmin-induced cleavage of chromogenic substrate S2251. Fibrinogen was incubated with either buffer only, anti-SUMO (sumo) or scaffold control proteins, or F5 at 1:1, 5:1, 10:1 Affimer:fibrinogen molar ratio prior to clotting. Clots were lysed by overlaying with tPA in the presence of S2251. **A.** Increase in absorbance readings for the first hour of plasmin generation assays. **B.** Average rates of S2251 hydrolysis for each sample tested in (A). **C.** Plasmin generation assay was also performed without fibrinogen, in the absence (buffer) and presence of F5 at 10:1. **D.** 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. **E.** In purified turbidity and lysis assays, pre-formed clots with and without Affimer protein were overlaid with either plasminogen (Plg) and tPA or plasmin. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA (B, D, E) or two-tailed Mann-Whitney test (C). ***$P \leq 0.001$ or ****$P \leq 0.0001$ represents difference from buffer only control. Data from Kearney et al. (2018).

4.2.14 Effects of fibrin on Affimer F5 action

Thus far, F5 has shown anti-fibrinolytic action in assays when present during clot formation, and when added to already-formed clots, indicating an ability to bind fibrinogen, and polymerised fibrin, respectively. When Affimer F5 was fibrinogen-bound (present during clot formation), it showed an ability to limit plasminogen to plasmin conversion (Figure 4-15). Plasmin generation assays in which the Affimer

was added to a pre-formed clot confirmed that the Affimer also had an ability to limit plasmin generation when fibrin-bound (Figure 4-16A,B).

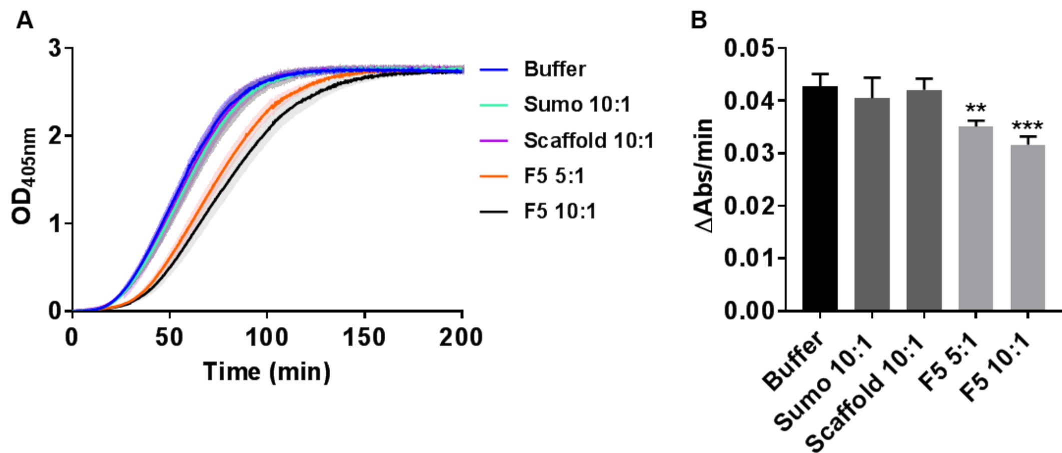


Figure 4-16 Mechanistic insights into Affimer F5-fibrin binding. Plasmin generation was assessed by monitoring hydrolysis of chromogenic substrate S2251. Samples contained purified fibrinogen and plasminogen, and were allowed to clot for 60 min prior to the addition of Affimer F5 at 5:1 or 10:1 Affimer:fibrinogen molar ratio, or anti-SUMO (sumo) or scaffold protein at 10:1. Affimers were added on top of formed clots, immediately before the addition of lysis mix containing tPA and S2251. **A.** S2251 hydrolysis over time. **B.** Average rates of S2251 hydrolysis for each sample tested in (A). n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ represents difference from buffer control.

4.2.15 Affimer F5 reduces plasmin generation through interference with tPA-plasminogen interaction

Given the fibrin(ogen)-specific reduction in plasmin generation by Affimer F5, and Affimer binding to the D region of fibrinogen, it was thought that the Affimer may function by directly blocking tPA or plasminogen access to fibrin(ogen). Competition assays were performed to determine if the Affimer could reduce binding of tPA and/or plasminogen to the fibrin DD fragment. Fibrin DD fragment was used due to the presence of multiple tPA and plasminogen binding sites within fibrin(ogen), making competition assays using whole fibrin(ogen) difficult to interpret. tPA, plasminogen and Affimer F5 all demonstrated binding to fibrin DD fragment, while scaffold protein showed no binding (Figure 4-17A,B,C,D). F5 did not reduce binding of tPA or plasminogen to DD fragment (Figure 4-17E,F), which suggests that the Affimer interferes with tPA-plasminogen interaction on the fibrin surface once these proteins are already fibrin-bound.

Additional control experiments confirmed that Affimer F5 showed no direct binding to either tPA or plasminogen (Figure 4-18A,B). Activity assays in purified systems (without fibrinogen) were also performed and confirmed that F5 had no direct effect on plasmin or tPA enzyme activity. The Affimer was incubated with increasing concentrations of tPA and plasminogen, prior to assaying tPA or plasmin protein activity using S2288 and S2251 chromogenic substrates, respectively. There was no significant effect of F5 on tPA or plasmin enzyme activity in these assays when compared with anti-SUMO control protein at each tPA or plasmin concentration used (Figure 4-18C,D).

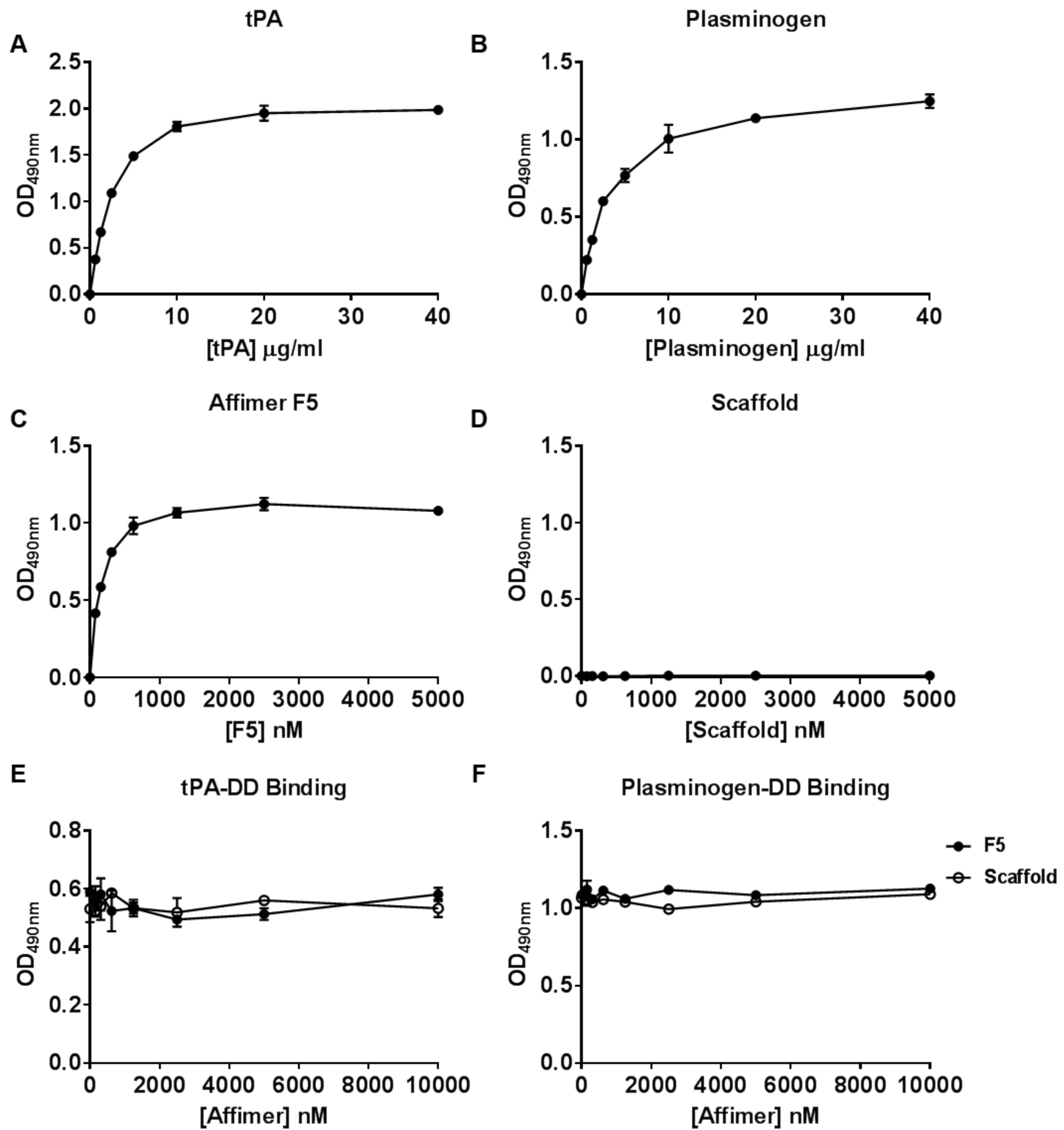


Figure 4-17 Binding of tPA and plasminogen to the fibrin DD fragment is not affected by Affimer F5. A. tPA **B.** plasminogen **C.** Affimer F5 **D.** Affimer scaffold binding to fibrin DD fragment was assessed by ELISA. Plates were coated with fibrin DD fragment and incubated with tPA, plasminogen, F5 or scaffold protein in concentration series. **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 (0-10,000 nM), and a constant concentration of tPA (1.25 µg/ml, 18 nM). tPA binding to DD was detected. **F.** A competition assay was performed similarly to that described in (E), with plasminogen (1.25 µg/ml, 14 nM) in place of tPA, and plasminogen detected. n=3, mean±SD of single representative experiments are presented. Data from Kearney et al. (2018).

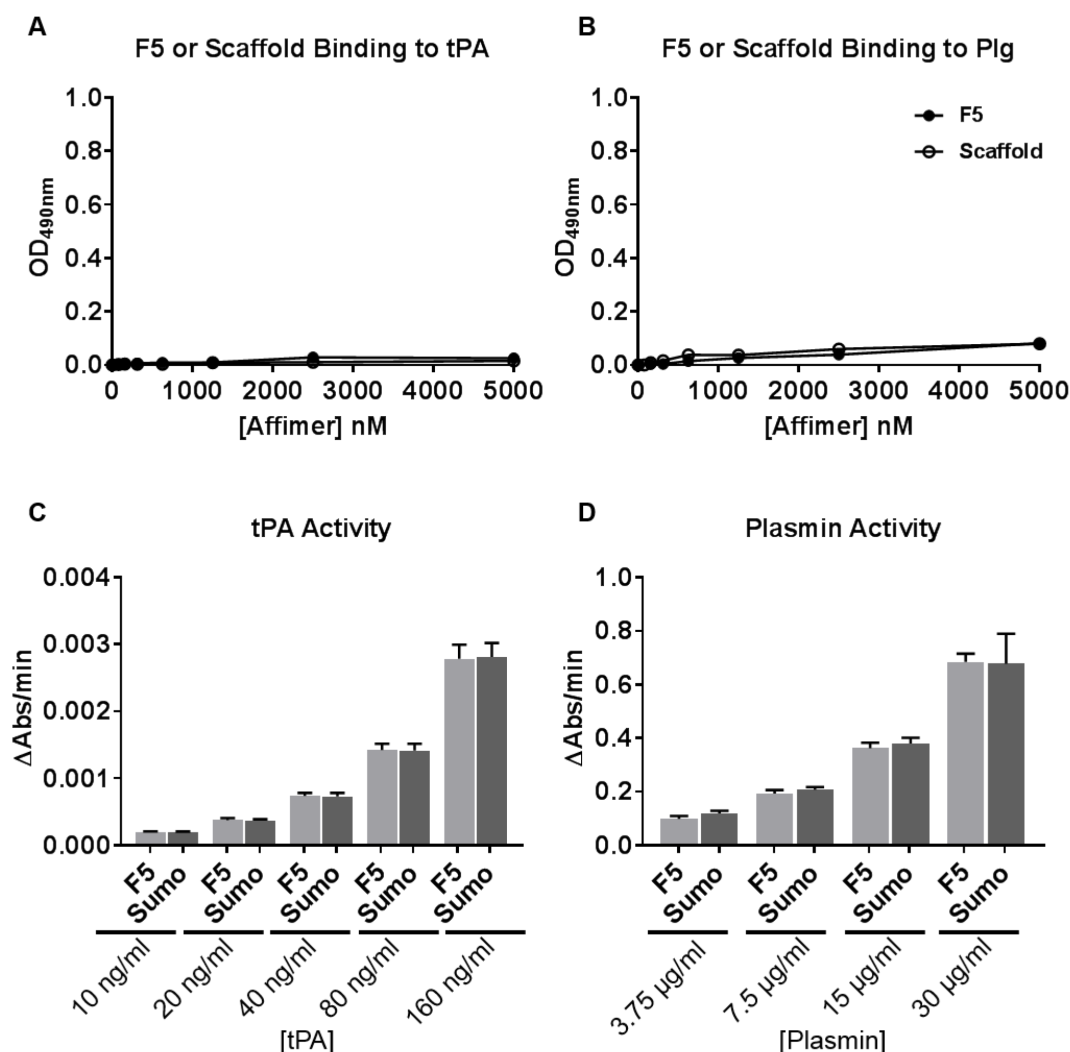


Figure 4-18 Affimer F5 does not directly bind to tPA or plasminogen, or affect their protein activity. A. Binding assays were performed to investigate any direct interaction of Affimer protein with tPA or **B.** plasminogen (plg). Plates were coated with (A) tPA or (B) plasminogen, and incubated with a concentration series of Affimer F5 or scaffold protein (0-5000 nM). F5 and scaffold protein were detected after incubation. n=3, mean±SD of single representative experiments presented. **C.** The effect of F5 on tPA enzyme activity was assessed in a purified assay, containing Affimer F5 or anti-SUMO (sumo) with increasing concentrations of tPA. Average rate of S2288 hydrolysis is presented. **D.** The effect of F5 on plasmin activity in a purified assay containing only Affimer F5 or anti-SUMO (sumo), and increasing concentrations of plasmin in the presence of chromogenic substrate S2251. Average rate of S2251 hydrolysis is presented. n=3, mean±SD. Statistical analysis was performed using two-tailed unpaired student *t*-test (C,D), comparing F5 with sumo control Affimer at each tPA or plasmin concentration. Data from Kearney et al. (2018).

4.2.16 Efficacy of Affimer F5 in mouse plasma

Animal models are necessary to show that Affimer proteins would be able to rapidly prevent blood loss and have potential as therapeutic agents. Turbidimetric assays using mouse plasma were performed with Affimer F5 to determine if there was inter-species reactivity of the Affimer. Figure 4-19 shows that the Affimer did not have an anti-fibrinolytic effect in mouse plasma, unexpectedly, the Affimer caused a small but significant enhancement of clot lysis. The inability to prolong lysis in mouse plasma suggests that F5 may interact with a different region of mouse fibrinogen than it does with human fibrinogen.

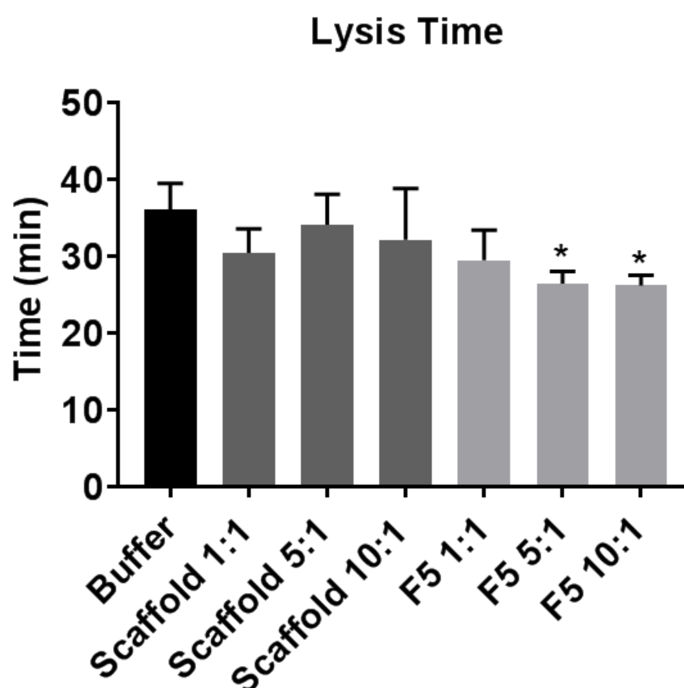


Figure 4-19 Turbidimetric assays with Affimer F5 and scaffold control protein in mouse plasma. F5 or Affimer scaffold were incubated with mouse plasma for 15 min, prior to the addition of tPA (225 ng/ml, 3.2 nM) for a further 20 min, and initiation of clotting with thrombin and calcium. Affimer protein was used at 1:1, 5:1 or 10:1 Affimer:fibrinogen molar ratio, buffer only control received no Affimer protein. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05 represents difference from buffer control.

4.3 Discussion

This chapter describes the characterisation of Affimer F5, a fibrinogen-binding Affimer with anti-fibrinolytic properties.

Affimer F5 was identified through phage panning against fibrinogen, and to characterise this binding interaction a combination of SPR and ELISA assays were used. These techniques demonstrated a high affinity interaction of the Affimer with fibrinogen which was similar to the Affimer's affinity for fibrin. These data suggest interaction of the Affimer with a region of fibrinogen which is similarly exposed in fibrin. There were some minor discrepancies between ELISA and SPR derived binding affinities, which may reflect the different adsorption on a plastic surface for ELISA versus derivatisation of the SPR dextran layer. Binding and immobilisation of a protein (in this case, fibrinogen) to a plastic surface in ELISA can denature the protein [340] and thus the Affimer-binding epitope. If the conformation of the fibrinogen is compromised, binding of Affimer to fibrinogen will also be compromised, resulting in a lower apparent affinity of the Affimer. In contrast, in SPR experiments fibrinogen was immobilised via amine coupling to the dextran surface, and thus with fewer points of contact between the immobilised protein and the surface it is possible that fibrinogen was better able to retain a native conformation. Retaining a native conformation would allow better recognition and binding by Affimer F5, reflected in the apparent higher affinity of F5 as determined by SPR compared with ELISA. Taken together, the binding studies showed that the Affimer bound similarly to fibrinogen and fibrin. Binding affinities differed between methods but reflected the high affinity of Affimer F5 for fibrin(ogen).

Despite screening Affimers against fibrinogen in order to isolate fibrinogen binders, it was important to determine the specificity of Affimer F5, and a pull-down assay was developed for this purpose. These assays confirmed that Affimer F5 could bind and pull fibrinogen out of plasma. In addition, a pull-down assay using F5 in plasma from afibrinogenemic patients showed that no additional proteins were pulled down by F5 in the (almost) absence of fibrinogen. The Affimer was in fact able to pull some fibrinogen from the a-fib patient plasma, demonstrating the Affimer's high binding affinity. However, caution must be used when interpreting pull-down identified binding partners, as these assays don't

identify weak or transient protein-protein interactions. Interestingly, pull-down assays followed by mass spectrometry identified kininogen-1 in Affimer pull-down elutions, a protein involved in initiation of blood coagulation in the intrinsic pathway [341]. This protein was identified as a potential binder to the Affimer scaffold backbone, as it was pulled down by control Affimers anti-SUMO and scaffold in addition to Affimer F5. Kininogen is made up of multiple protein domains, including three cystatin domains [342] and so it is possible that there is interaction of the Affimer, the scaffold of which is based on cystatin protein, with the cystatin domains of kininogen. Formation of oligomers by cystatin proteins via domain swapping has been reported [343-345]. However, high molecular weight kininogen contains a histidine-rich fragment, in which histidine residues are non-consecutively placed [346], and it may be that this histidine rich fragment is causing kininogen to be pulled down (non-specifically) by his-tag affinity beads [347]. In support of this, in pull down assays using HisPur beads (not Dynabeads), there is noticeably less protein in the region where kininogen is detected in Dynabead pull down elutions. There are clearly multiple possible explanations for the presence of kininogen in Affimer pull-down elutions, and is an area for further investigation to determine if this protein-protein interaction is a specific one. Together these data demonstrated that Affimer F5 was able to bind to fibrinogen in solution and that the Affimer is selective for fibrinogen.

Affimer F5 showed an ability to prolong lysis time in a plasma system without significantly altering lag time or maximum OD. In a purified system F5 retained its effect on the prolongation of lysis time but increased lag time and reduced maximum OD. While the effects of the Affimer on clot lysis are consistent, lag time and clot maximum OD differ in plasma and purified protein experiments. A reduced maximum OD in a purified system suggests the formation of thinner fibres or a less dense clot [166, 319, 348], although interpretation of turbidimetric assay-derived maximum OD is contentious. Fibres were thicker in the presence of the Affimer when viewed by SEM, and the formation of a less-dense clot was confirmed by SEM, confocal and permeation experiments.

The increase in fibre thickness with the Affimer as determined by SEM suggested that F5 facilitated lateral aggregation although this is unexpected given the binding of the Affimer to the D fragment, as it is the fibrinogen αC regions that are

associated with lateral aggregation of protofibrils [21]. Moreover, molecular modelling predicted binding of the Affimer close to the C-terminal end of the γ chain in the D fragment, close to where the E region of adjacent fibrin molecules would align during formation of protofibrils. It seemed more likely that the Affimer would disrupt fibre formation by binding to this site, but this was not the case. Although studies suggest that clots with thinner fibres take longer to lyse, this is usually due to the formation of more compact clots with thinner fibres (hence having more fibres to lyse). Studies investigating lysis of individual fibrin fibres have shown that on a single fibre level, thicker fibres take longer to lyse, and therefore one mechanism for prolongation of lysis by F5 could be related to the formation of thicker fibres [131, 132]. Looking at the effect of F5 on clot density, F5, when tested at 5:1 Affimer:fibrinogen molar ratio in chapter 3 (section 3.2.7) did not show any significant effect on clot density as viewed by confocal microscopy. However, in chapter 4 (section 4.2.6), the Affimer was reported to cause a small, but statistically significant reduction in clot density at 5:1 and 10:1 Affimer:fibrinogen molar ratios. The discrepancy between the effects seen at 5:1 are likely due to natural heterogeneity between Affimer protein batches. The formation of a more porous clot by F5 is unexpected, as typically, clots that are more porous are more susceptible to degradation, due to increased permeability to lytic enzymes. Therefore, these data indicated that changes to clot structure were not solely responsible for the prolongation in lysis time by F5.

Further experiments confirmed that the mechanism of action of F5 was not dependent on alteration of fibrin clot structure. Turbidimetric experiments and plasmin generation assays in which F5 was added to a pre-formed clot showed an ability of the Affimer to prolong fibrinolysis, secondary to reduced plasmin generation. These data suggested an interaction of the Affimer with the actions of fibrinolytic enzymes tPA and/or plasminogen. Taken together, experimental findings suggest that Affimer F5 causes minor alterations to clot structure when present during clot formation, but that it has a more complex means of delaying fibrinolysis than changes to structure alone. The ability of the Affimer to act via fibrinogen- or fibrin- binding raised questions about the location of Affimer-fibrin(ogen) binding sites.

A combination of pull-down experiments and molecular modelling was used to identify an interaction of Affimer F5 with the fibrinogen D fragment. Molecular modelling simulations using the crystal structure of fragment D pinpointed the γ chain of fibrinogen to be a potential binding site for the Affimer. Docking studies predicted interaction of F5 in close proximity to the γ312-324 tPA and the α148-160 tPA/plasminogen binding site [87, 91, 92, 349]. Although the latter site binds tPA and plasminogen with similar affinity (~K_D 1 μM) [89], it would be saturated with plasminogen under physiological conditions due to the molar excess of plasminogen over tPA in the circulation [83]. Plasmin generation therefore follows interaction of tPA with plasminogen at these D region sites. Binding studies indicated that Affimer F5 did not alter binding of tPA or plasminogen to fibrin(ogen), at least to DD fragment. Given the fibrin dependent mode of F5, the most likely explanation is that Affimer F5 interferes with tPA-plasminogen interaction once these proteins are bound to the fibrin surface. This concept is supported by the molecular modelling data which show the Affimer binding fibrinogen in an area spatially situated between tPA and plasminogen binding sites in the D fragment. Furthermore, since binding studies and activity assays determined the Affimer did not directly bind to or limit the activity of plasmin or tPA, this fibrin-dependent interference with plasminogen-tPA is the most plausible mechanism of action. Further definitive work would be required to confirm this mechanism of action, which is perhaps best conducted using crystallography studies of fibrin(ogen) in complex with the Affimer and tPA/plasminogen. What is clear is that the mode of action of the Affimer depends upon constraint of the variable region peptides within the Affimer scaffold, as synthetic peptides mimicking the Affimer variable regions did not have any effect on clot lysis in turbidimetric assays. Molecular modelling demonstrated that the 9 amino acid sequences adopted conformations which were linear and highly flexible when isolated short peptides, in contrast to their conformation when a part of the Affimer scaffold.

Binding of Affimer F5 to the fibrinogen D fragment at the sites predicted by molecular modelling put the Affimer in close proximity to FXIII crosslinking sites located near the C-terminus of the γ chain [350]. Comparison of fibrin degradation products formed from the lysis of clots made with and without Affimer F5 showed a reduced number of crosslinked γ species in clots made with F5. While this may

simply reflect the slower release of crosslinked species from clots made with F5 due to a delay in fibrinolysis, there were also more uncrosslinked γ chains in clots made with the Affimer. These uncrosslinked γ chains were not present at early lysis time points, but appeared after the clots began to lyse. This finding suggests that there were more uncrosslinked γ chains in clots made with F5, which became liberated as the clots were digested with plasmin. Additionally, γ', a product of the digestion of uncrosslinked γ chain, was only present in the lysis products of clots made with F5, and not in those clots made without the Affimer. These data suggest an effect of the Affimer on FXIII crosslinking, however, FXIII crosslinking is generally associated with increased resistance to lysis, not increased susceptibility, although reports are conflicting. Standeven et al. (2007) found that FXIII crosslinking did not delay fibrinolysis [350], others have shown the converse [22, 23, 351], and some report that FXIII only increases clot stability through the crosslinking of plasmin inhibitor into clots [120]. Increased fibre thickness in the presence of F5 also points to reduced FXIII crosslinking in clots formed with the Affimer, as FXII crosslinking is associated with reduction in fibre thickness [22, 352]. Taken together, experimental data suggests that Affimer F5 may limit FXIII crosslinking of fibrinogen γ chains, due to the interaction of the Affimer with the C-terminal region of the γ chain. However, any effect on crosslinking is likely a side-effect of the Affimer-fibrinogen binding site, and not related to the effects of the Affimer on prolongation of clot lysis. A FXIII crosslinking gel, as described elsewhere [353], is needed to determine with certainty if there is an effect of the Affimer on the ability of FXIII to crosslink γ chains during fibrin polymerisation.

Prolonging clot lysis, and thus stabilising the fibrin network offers the opportunity to treat bleeding typically seen following surgery or trauma as well as in some haematological abnormalities affecting clotting factors. Affimer F5 showed a consistent effect on prolongation of lysis when used in plasma from healthy individuals, and in plasma deficient in FVIII (mimicking the factor deficiency in individuals with haemophilia A). Affimer F5 successfully reversed the pathological enhancement of clot lysis in FVIII deficient plasma. This would be particularly helpful in more challenging cases where haemophilia patients develop antibodies against FVIII, which limit the usefulness of coagulation protein replacement [354, 355]. It would be beneficial to test the Affimer for an ability to normalise fibrinolysis in plasma from patients with other types of coagulation deficiencies typically

associated with bleeding, such as FXIII deficiency [356], and is an area for further research.

Affimer F5 was able to prolong clot lysis time in whole blood of healthy volunteers, demonstrating that the presence of blood cells did not hinder the protein's anti-fibrinolytic actions. The Affimer did not significantly reduce maximum clot firmness (MCF), and so, if F5 does reduce γ chain crosslinking by FXIII, it does not significantly impact clot strength in whole blood [322]. In addition to FXIII crosslinking, platelet count and platelet function also contribute to MCF [322]. Whole blood flow cytometry and aggregation assays confirmed that the Affimer had no effect on platelet activation or aggregation, consistent with the absence of an effect of the Affimer on MCF in ROTEM. However, F5 caused a significant prolongation of clotting time (CT) and clot formation time (CFT) in whole blood. CT and CFT in ROTEM are analogous to lag time in turbidimetric assays, as they represent the initial stages of clot formation, and early fibrin polymerisation. Affimer F5 caused a prolongation of lag time in purified turbidimetric assays, and in plasma from healthy individuals, but not in pooled plasma experiments. These data suggest that the Affimer may have an effect on initiation of clotting although it is hard to reconcile the findings from assays in different systems. This highlights a need for further work to determine the mechanism by which the Affimer is altering these clotting parameters in whole blood.

A strength of this study is the development of a novel methodology to stabilise the fibrin clot that may have future clinical implications. The increase in clot lysis time by F5 ranged from 2-10 fold, depending on the concentration of the Affimer and the system used to study the Affimer effect. Recent studies in bleeding and thrombotic disorders indicate that less than 50% difference in clot lysis time can be clinically significant [357-360] and therefore the effects of Affimer F5 are meaningful for future clinical use. A further strength of this particular Affimer is its fibrin-dependent mode of action, confirmed by the absence of an effect when uPA was used to initiate clot lysis [339]. This is a particular strength as any unwanted thrombotic event with clinical use of F5 could be managed with fibrinolytic agents that convert plasminogen to plasmin independent of fibrin, such as urokinase and streptokinase. A constant difficulty in developing anti-coagulant or clot stabilising agents is the absence of an "antidote" in the event of bleeding or thrombotic

complications, respectively. Given the mode of action of F5, there already exist reversing agents should unwanted thrombosis occur during future *in vivo* use.

From the clinical point of view, Affimer proteins may be used directly or alternatively may provide a tool for identification of new therapeutic targets that can be subsequently modulated using small molecules. This study has shown that Affimer technology can be used to modulate fibrinolysis whilst preserving physiological clot structure and is effective at normalising lysis time in conditions associated with increased bleeding risk. However, there are limitations to this work, including the limited number of fibrinogen-binding Affimer proteins isolated during screening (described in chapter 3). Larger screens are required, including investigation of Affimer proteins from earlier panning rounds and with lower affinities, which will provide a larger number of Affimer proteins that may be more suitable for clinical use. Additionally, whilst *ex vivo* studies show a consistent effect for F5, which is promising, *in vivo* work is yet to be conducted using animal models of bleeding. Studies using Affimer F5 in mouse plasma showed that the Affimer had no anti-fibrinolytic ability in this system, presumably because whilst the mouse fibrinogen γ chain exhibits ~80% homology with the human fibrinogen γ chain, the Affimer must interact in a different region of mouse fibrinogen than in human fibrinogen. Unexpectedly, the Affimer enhanced lysis in mouse plasma, an effect which cannot be explained without further investigation. It may be that F5 induces structural changes in mouse plasma similarly to its effect in human plasma, with the formation of thicker fibrin fibres, and networks which are less dense, resulting in the formation of clots which are easier to lyse. The absence of a prolongation in lysis by the Affimer in mouse plasma currently remains a mystery because plasminogen and tPA binding sites on mouse fibrinogen have no yet been characterised. Given the proposed mechanism of action of Affimer F5, the Affimer binding site on mouse fibrinogen would need to be located between plasminogen and tPA binding sites, allowing the Affimer to block the interaction of these proteins on the fibrin surface. Without knowing F5, tPA and plasminogen binding sites on mouse fibrinogen these theories cannot currently be explored further. The isolation of Affimers which have a consistent effect in human and mouse plasma is needed to allow investigation of the *in vivo* safety and efficacy of Affimers in future studies.

In conclusion, this work provides a proof of concept study of a novel methodology to alter fibrinolysis and stabilise the fibrin clot. The ability to stabilise a fibrin clot with Affimer proteins may offer a simple and affordable way to limit bleeding following traumatic vessel injury or in pathological conditions with inherent abnormalities in the fibrin networks. Future work should concentrate on isolating a larger number of fibrinogen-binding Affimer proteins. Additionally, future *in vivo* animal experiments will be required using Affimers that show activity with both human and mouse proteins before Affimers can be considered for clinical development.

Chapter 5 Modulation of fibrinolysis with fibrinogen-binding type 1 Affimers

5.1 Introduction

Chapters 3 and 4 described the isolation and characterisation of type II fibrinogen-binding Affimers. This chapter describes type I fibrinogen-binding Affimers isolated in collaboration with Avacta Life Sciences Ltd. The purpose of the screen was to identify fibrinogen binders which could modulate fibrin clot lysis (as described in chapter 4) and subsequently to characterise their functions in a variety of systems and identify mechanisms of action.

The type II Affimer scaffold is based on a consensus sequence of a cystatin from a number of plant species [289], whereas type I Affimers have a scaffold based on the naturally occurring human protease inhibitor Stefin A [306]. Both types of scaffold have a similar tertiary structure, despite sharing only around 30% sequence identity. The benefit of utilising an Affimer scaffold based on a human protein is the potential for minimised immunogenicity if Affimers were to proceed to human trials in the future.

In this chapter I describe the process of screening humanised Affimers isolated by Avacta for an effect on fibrinolysis, and subsequent characterisation of Affimer B3. The following studies have provided useful insight to inform future work with Affimers.

5.2 Results

5.2.1 Isolation and selection of fibrinogen-binding Affimers

Six unique type I fibrinogen-binding Affimers were isolated following three rounds of phage panning by Avacta.

The Affimers were screened for an effect on plasma clot lysis in turbidimetric assays using increasing concentrations of the Affimers (Figure 5-1). Affimer B3 increased lysis time from 24.7±9.8 min in buffer only control to 47.9±9.5 min ($P \leq 0.05$) at 5:1 Affimer:fibrinogen molar ratio and 51.3±24.5 min ($P \leq 0.01$) at 10:1 (mean±SD). Lysis time for Affimer B3 could not be calculated at 20:1 due to low turbidity readings in this sample (turbidity readings did not reach 0.01 AU). Affimer B9 also increased lysis time, to 54.5±4.4 min ($P \leq 0.01$) at 10:1 and 102.3±8.1 min (mean±SD) ($P \leq 0.001$) at 20:1 Affimer:fibrinogen molar ratio (Figure 5-1).

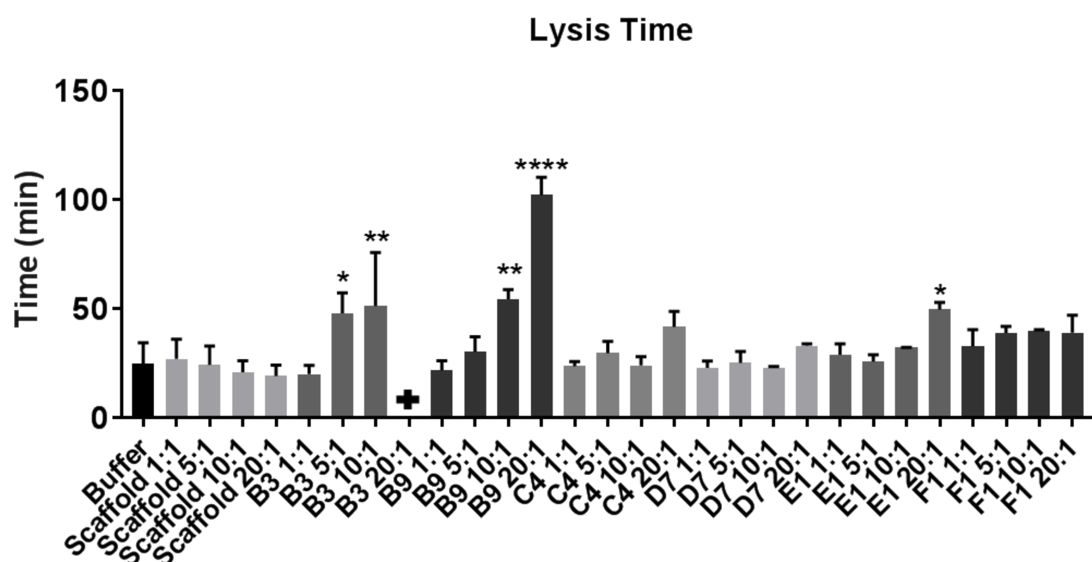


Figure 5-1 Screening of type I fibrinogen-binding Affimers in a plasma turbidity and lysis assay. Pooled human plasma was incubated with Affimers at 1:1, 5:1, 10:1 and 20:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin, calcium and tPA. A plasma sample receiving buffer only (no Affimer protein) was used as a control. .✚ Indicates that turbidity readings were too low to calculate lysis time when B3 was used at 20:1. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ represents difference from buffer only control.

Affimers B3 and B9 were chosen for further study due to their significant prolongation of clot lysis time. The amino acids in the variable regions of the two Affimers displayed some similarities. For example, R (arginine) and E (glutamic acid) in B3 and B9, respectively, both have electrically charged side chains; H

(histidine) and R (arginine) have positively charged side chains; and F (phenylalanine) and H (histidine) are aromatic. Figure 5-2 shows the Affimers in a sequence alignment, with the variable regions of each underlined in purple.

```
B3              1 MMPRGLSEAKPATPEIQEIVDKVKPQLEEKTNETYGKLEAVQYKTQVLAL     50
                    |||||||||||||||||||||||||||||||||||||||||||||||||.
B9              1 MMPRGLSEAKPATPEIQEIVDKVKPQLEEKTNETYGKLEAVQYKTQVLAP     50

B3             51 *******STNYYIKVRAGDNKYMHLKVFNGPQ*******ADRVLTGYQ    100
                  .......||||||||||||||||||||||||.:.......:|||||||||
B9             51 *******STNYYIKVRAGDNKYMHLKVFNGPY*******ADRVLTGYQ    100

B3            101 VDKNKDDELTGFGHHHHHH    119
                  |||||||||||||||||||
B9            101 VDKNKDDELTGFGHHHHHH    119
```

Figure 5-2 Sequence alignment of Affimers B3 and B9. Affimer protein sequences were aligned using EMBOSS Needle online sequence alignment service. The two 9 amino acid variable regions are indicated with purple lines. A colon (:) indicates amino acids at that position that have strongly similar properties, while a full stop (.) indicates amino acids at that position that have somewhat similar properties according to EMBOSS Needle alignment algorithm. Due to potential future patent considerations, only the first amino acid sequences of the Affimer variable region loops are shown.

Two types of control Affimers were used in experiments, named SQT His and SQT Gly. SQT His is the type I scaffold without any variable region inserts, and is engineered to be able to accept a peptide library. SQT Gly is the type I human scaffold with 9 glycine residues in each of the two variable regions. SQT Gly is used in addition to SQT His because its molecular weight more closely resembles that of the Affimers with the variable region inserts. Where “scaffold” is referred to, SQT His protein has been used, and where both scaffolds are used they are named accordingly. Both types of control Affimers were shown to have no significant effect on lag time, maximum OD, or lysis time when tested in plasma (Figure 5-3A) and purified (Figure 5-3B) systems in turbidimetric assays.

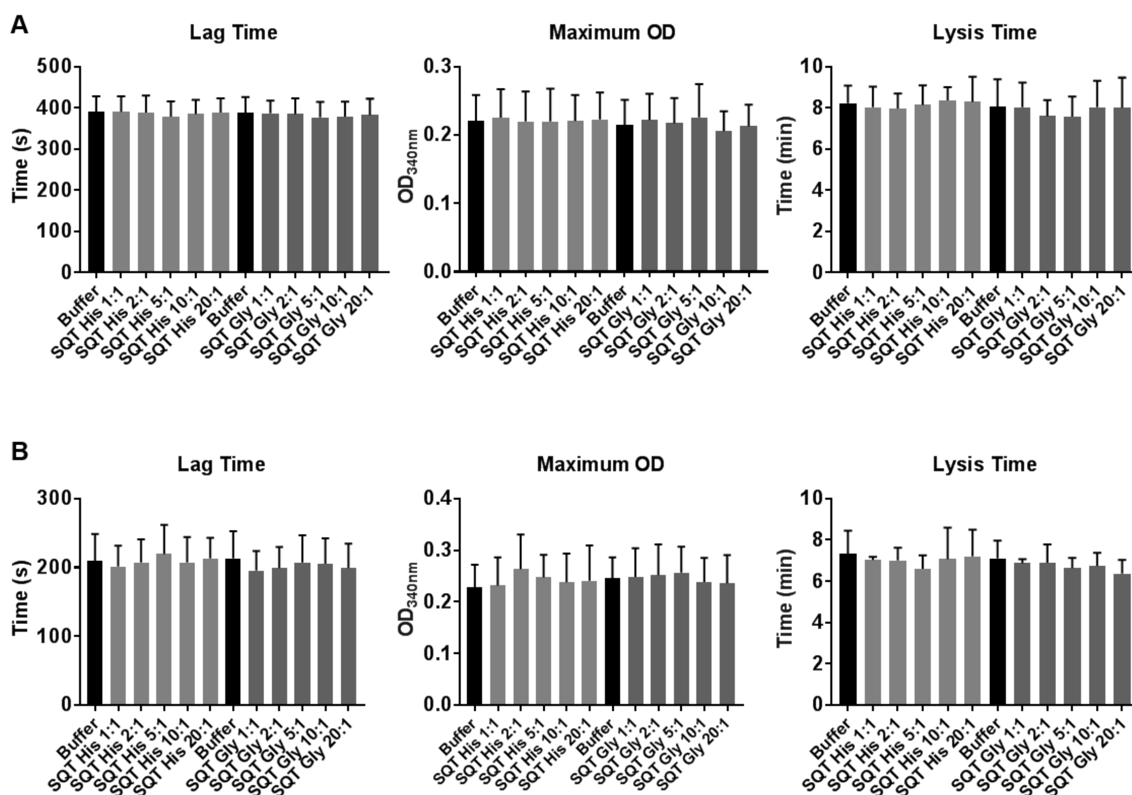


Figure 5-3 Effect of control Affimers SQT His and SQT Gly on clot formation and lysis in plasma and purified systems. Pooled human plasma or fibrinogen was incubated with Affimers at 1:1, 2:1, 5:1, 10:1 and 20:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays), and thrombin, calcium and tPA (turbidity and lysis assays). Buffer only samples containing no Affimer protein were used as controls. **A.** (Left to right) lag time, maximum OD and lysis time in a plasma system. **B.** (Left to right) lag time, maximum OD and lysis time of scaffolds in a purified system. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, comparing each molar ratio of Affimer used to the buffer only control.

5.2.2 The effect of fibrinogen-binding Affimers B3 and B9 on clot formation and lysis in plasma

B3 and B9 were used in turbidimetric assays in plasma to assess their effect on clot properties in more depth following the preliminary screens used to select the Affimers. B3 significantly increased lag time at 5:1, 10:1 and 20:1 Affimer:fibrinogen molar ratio, while B9 caused an increase in lag time at 20:1 (Figure 5-4A). B3 significantly decreased maximum OD at 5:1, 10:1 and 20:1 with B9 showing similar effects at 10:1 and 20:1 (Figure 5-4B). B3 increased lysis time at 5:1, 10:1 and 20:1, while B9 caused an increase at 10:1 and 20:1 (Figure 5-4C). Results are summarised in Table 5-1.

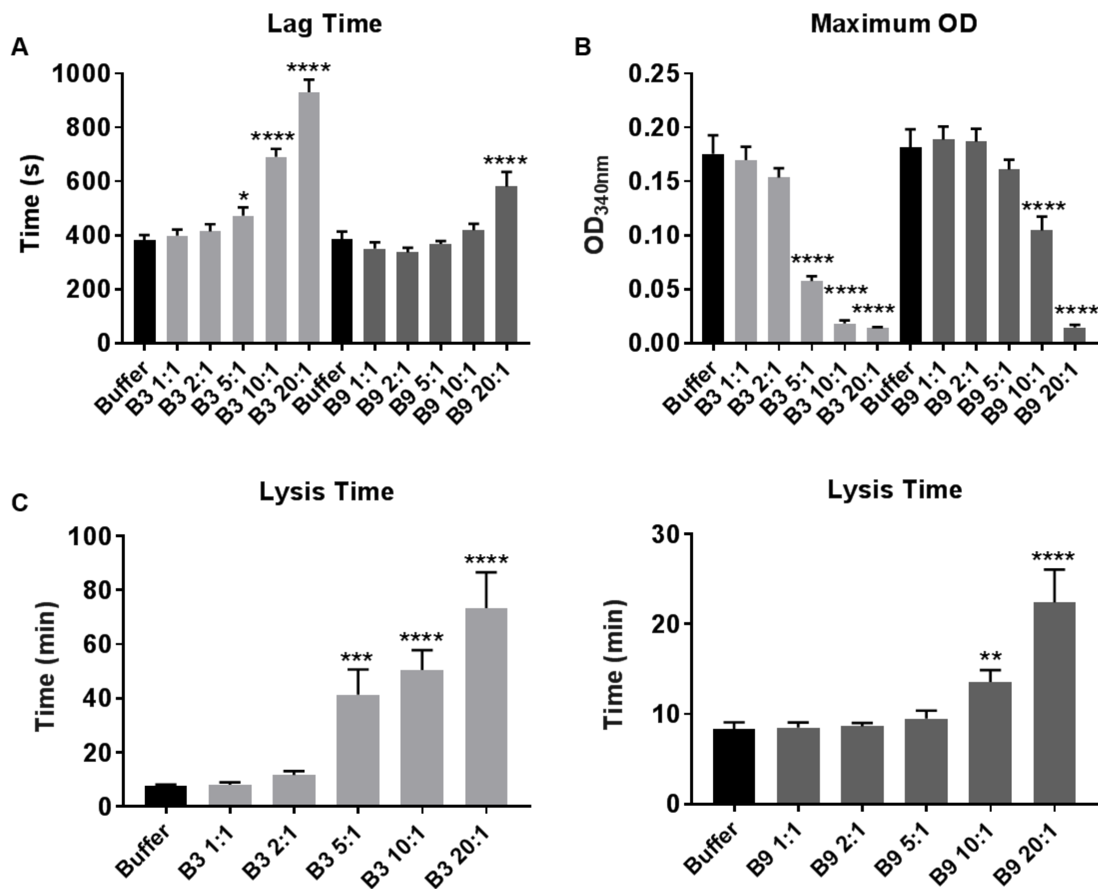


Figure 5-4 Effect of increasing doses of Affimers B3 and B9 on clot formation and lysis in plasma. Pooled human plasma was incubated with Affimer at 1:1, 2:1, 5:1, 10:1 and 20:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays), and thrombin, calcium and tPA (turbidity and lysis assays). Samples receiving buffer only (no Affimer protein) were used as controls. **A.** Lag time **B.** Maximum OD for Affimers B3 and B9. **C.** Lysis time for Affimers B3 (left) and B9 (right). n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 represents difference to buffer only control.

Affimer:fibrinogen molar ratio	Lag time (s, mean±SD)	Maximum OD (AU, mean±SD)	Lysis time (min, mean±SD)
Buffer only	382.7±18.8	0.1755±0.0171	7.7±0.4
B3 1:1	398.7±22.2	0.1697±0.0123	8.1±0.9
B3 2:1	414.7±26.3	0.1534±0.0088	11.7±1.4
B3 5:1	472.7±31.3*	0.0577±0.0046****	41.4±9.3***
B3 10:1	690.7±29.7****	0.0184±0.0029****	50.5±7.3****
B3 20:1	930.7±46****	0.0145±0.0007****	73.3±13.3****
Buffer only	384.7±28.7	0.1813±0.0170	8.3±0.8
B9 1:1	350.7±24.1	0.1887±0.0120	8.5±0.6
B9 2:1	336.7±17.2	0.1867±0.0118	8.6±0.4
B9 5:1	368.7±10.0	0.1610±0.0090	9.5±0.9
B9 10:1	418.7±24.6	0.1044±0.0131****	13.6±1.3**
B9 20:1	580.7±53.4****	0.0146±0.0025****	22.4±3.7****

Table 5-1 The effects of Affimers B3 and B9 on clot formation and lysis parameters in plasma. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 represents difference to buffer only control.

5.2.3 The effect of Affimers B3 and B9 on clot structure in plasma

Given the alterations in clot turbidity following treatment of plasma samples with Affimer B3 and B9, changes in clot structure were investigated using confocal and SEM.

Confocal microscopy experiments were performed to investigate the 3D structure of hydrated clots formed in the presence of Affimers. Control Affimers SQT His and SQT Gly caused no significant morphological changes to clot structure when compared with buffer only control and no significant changes to fibre count (Figure 5-5A,B,C). In contrast, addition of Affimers B3 and B9 to clots resulted in the formation of clots that were more dense, with more fibres and smaller pores (Figure 5-5A), such that image analysis software was unable to count the number of fibres in the confocal images. Changes to clot density are visually evident in B3 at all concentrations used, while B9 caused a visible change to clot density at the highest concentration used of 10:1.

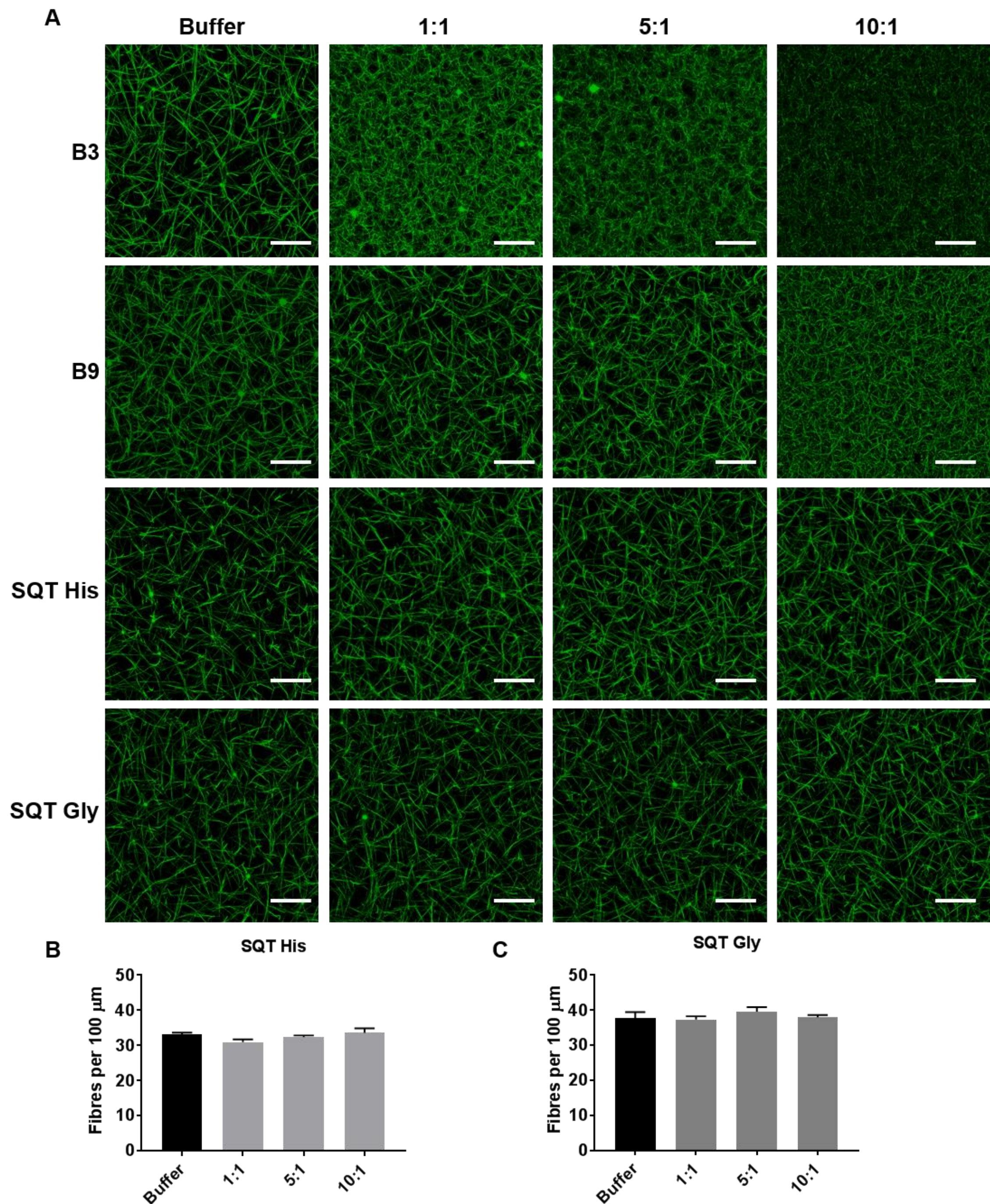


Figure 5-5 The effect of Affimers on clot structure in plasma. A. Representative confocal images of Affimer B3, B9 and scaffold in pooled human plasma at 1:1, 5:1 and 10:1 Affimer:fibrinogen molar ratio. Buffer only samples received no Affimer protein. Clots were made with the addition of fluorescently labelled Alexa Fluor-488 fibrinogen. Z stacks of 30 images over 20.30 µm were taken, 3D images of compiled Z stacks are presented. **B.C.** Average fibre count of plasma clots formed in the presence of **B.** SQT His and **C.** SQT Gly. Two clots were made for each condition, and 3 images taken in different areas of each clot. Fibre density was determined by counting the number of fibres that cross 20 lines across each image, using ImageJ software. Data presented as mean±SD. Statistical analysis was performed using one-way ANOVA, comparing each molar ratio of Affimer used to buffer only control. Scale bar = 20 µm.

Confocal data showing increased clot density (and so, decreased clot porosity) were confirmed with permeation experiments, performed by Dr Ramsah Cheah. In these experiments, plasma clots were formed in the presence of Affimers and the movement of assay buffer through the clots measured over time. The Darcy constant, which describes the movement of fluid through a porous medium, was significantly decreased for clots formed with B3 and B9, at both 5:1 and 10:1 Affimer:fibrinogen molar ratios (Table 5-2). Clots formed with scaffold control protein did not have significantly altered permeation properties.

Sample	n	Ks ± SD (10^{-9} cm^2)
Buffer	8	5.01 ± 0.77
Scaffold 5:1	5	4.46 ± 0.79
Scaffold 10:1	3	4.70 ± 0.82
B3 5:1	8	2.50 ± 1.30***
B3 10:1	6	2.30 ± 1.50***
B9 5:1	7	2.40 ± 0.24***
B9 10:1	6	2.20 ± 1.30***

Table 5-2 The effects of Affimers on the permeation properties of plasma clots. Clots were made in the presence of buffer only, scaffold protein, and Affimer B3 and B9 at 5:1 and 10:1 Affimer:fibrinogen molar ratio in pooled human plasma. The Darcy constant (Ks) of each sample was calculated using the flow measurements acquired. Data presented as mean±SD. Statistical analysis was performed using one-way ANOVA, *** P≤0.001 represents difference to buffer only control.

Confocal microscopy analysis of clots formed in the presence of B3 and B9 enabled the 3D structure to be viewed in hydrated conditions. However, in order to investigate clot ultrastructure in the presence of Affimers, clots were prepared for SEM which involved step-wise dehydration of the clots. In contrast to confocal microscopy experiments, an effect of the Affimers on clot density was not detected in SEM images, however, Affimers B3 and B9 both caused a reduction in fibre thickness. In B3 samples, there was a small but statistically significant decrease in fibre thickness from 103.1±6.6 nm to 86.4±11.8 nm at 5:1 (P≤0.05) and 80.1±6.5 nm (P≤0.01) at 10:1 (mean±SD) (Figure 5-6A,B). In B9 samples, fibre thickness was reduced from 75.9±12.9 nm in buffer only controls to 58.2±6.3 nm at 5:1 (mean±SD) (P≤0.05) (Figure 5-6A,C).

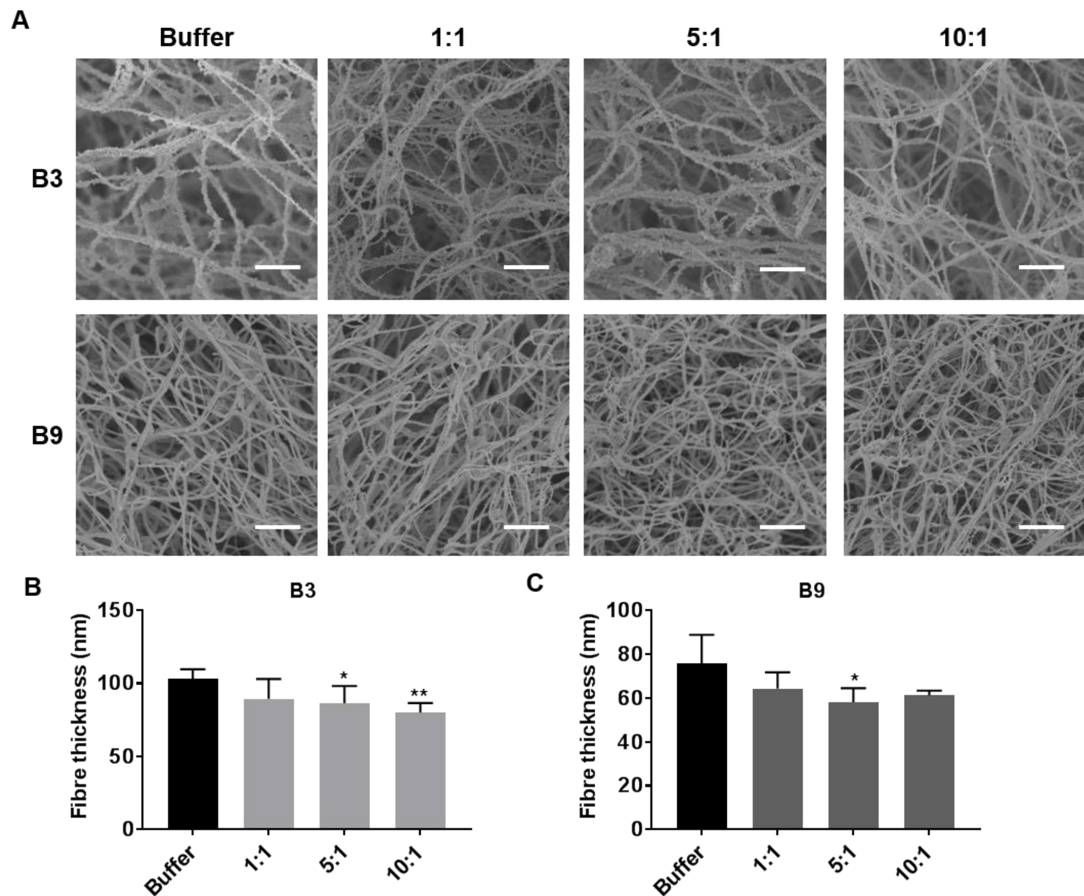


Figure 5-6 SEM images of plasma clots formed in the presence of Affimer B3 and B9. Affimers were incubated with plasma at 1:1, 5:1 and 10:1 Affimer:fibrinogen molar ratio. Plasma samples receiving buffer only were included as controls. **A.** Representative SEM images of clots formed in the presence of Affimers B3 and B9, scale bar = 1µm. **B.** Mean fibre thickness (nm) of fibres in SEM images. For each concentration of Affimer used, 2 clots were made, and images taken in 5 different areas of each clot. To determine fibre thickness, 15 fibres were measured in each area taken at 10K magnification. Data presented as mean±SD. $*P \leq 0.05$, $**P \leq 0.01$ when compared with buffer only control using one-way ANOVA.

5.2.4 Affimer B9 does not function as a monomeric protein

The type I Affimers were isolated from the phage library by their ability to bind immobilised fibrinogen, however, it was of interest to confirm fibrinogen binding in solution and identify any other possible Affimer-plasma protein interactions. A pull down assay in plasma was performed for this purpose. Affimer protein was captured with Dynabeads and incubated with plasma, prior to washing and elution of bound protein from the Dynabeads. Figure 5-7A shows pulldown elutions of Affimer B9 and the type I scaffold SQT His. B9 pulled fibrinogen out of plasma, confirmed as such in a western blot using a polyclonal anti-fibrinogen antibody (Figure 5-7B). B9 pull down elutions also contained 7 bands which ran below the γ chain of fibrinogen on SDS-PAGE gels (Figure 5-7C, red arrows). These 7

bands were sent for identification by mass spectrometry, and identified as containing primarily *E. coli* proteins, in addition to detection of Affimer protein multimers in some of the bands. The bacterial proteins were presumably present from the bacterial expression system used to produce the Affimer protein.

The B9 pull down elutions (Figure 5-7C) were also analysed by SDS-PAGE alongside batches of Affimer B9 protein grown in Leeds and those grown by Avacta. This gel (Figure 5-7D) revealed that the contaminating protein bands seen in B9 pulldown elutions were present in all batches of Affimer protein, in addition to other contaminating bands (Figure 5-7D). Following re-growth of Affimer B9 by Avacta, and SEC to further purify the protein, two pools of SEC elutions, one more pure than the other (Figure 5-8A), were tested for their functionality. When compared with a previous (active) batch of Affimer B9 protein (Figure 5-8B), the less-pure Affimer sample behaved comparatively (Figure 5-8C), however, the pure, monomeric form of the Affimer did not show any of the same effects on clotting/lysis (Figure 5-8D). Due to the lack of any functionality of the Affimer in the absence of multimers and contaminant proteins, no further investigation into B9 was performed.

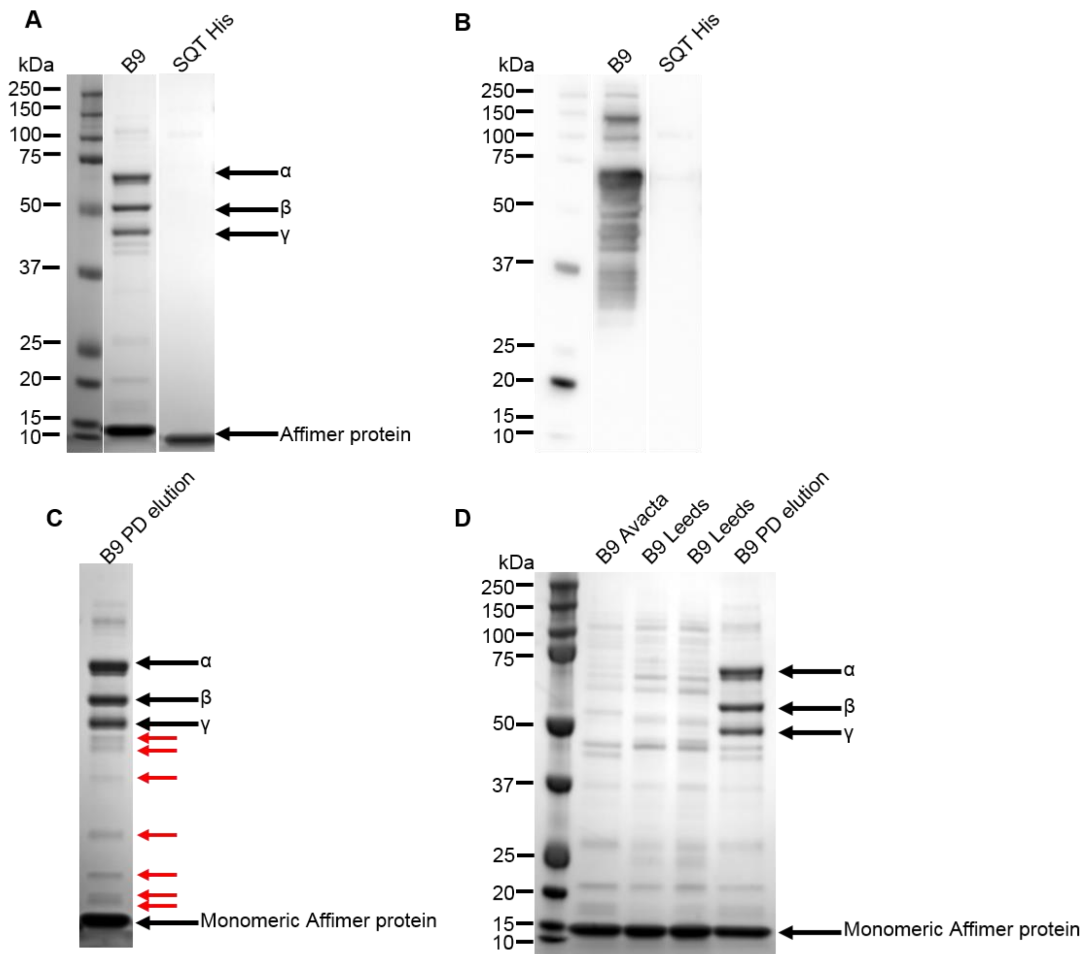


Figure 5-7 Pull down assays with B9 and SQT His scaffold control protein to determine the fibrinogen specificity of Affimers. B9 and SQT His were incubated with Dynabeads to allow the C-terminal his-tag of the Affimers to adhere to the Dynabead surface. Dynabeads were then incubated with plasma for 30 min prior to washing, and elution of proteins from the Dynabeads. **A.** Pull down elutions were analysed by SDS-PAGE. The position of the Affimer proteins, and the three chains of fibrinogen are indicated. **B.** Anti-fibrinogen blot of pull down elutions (shown in A) using a polyclonal anti-fibrinogen antibody. **C.** B9 pull down (PD) elution was re-run on a gel, and the bands indicated by red arrows were identified by mass spectrometry as being composed primarily of *E. coli* proteins and aggregates of Affimer protein. **D.** Aliquots from all batches of B9 protein were analysed by SDS-PAGE which confirmed that the contaminating bands seen in pull down (PD) elutions were also present in all B9 protein produced to-date. Labels above each lane indicate the source of the protein (Avacta or Leeds grown).

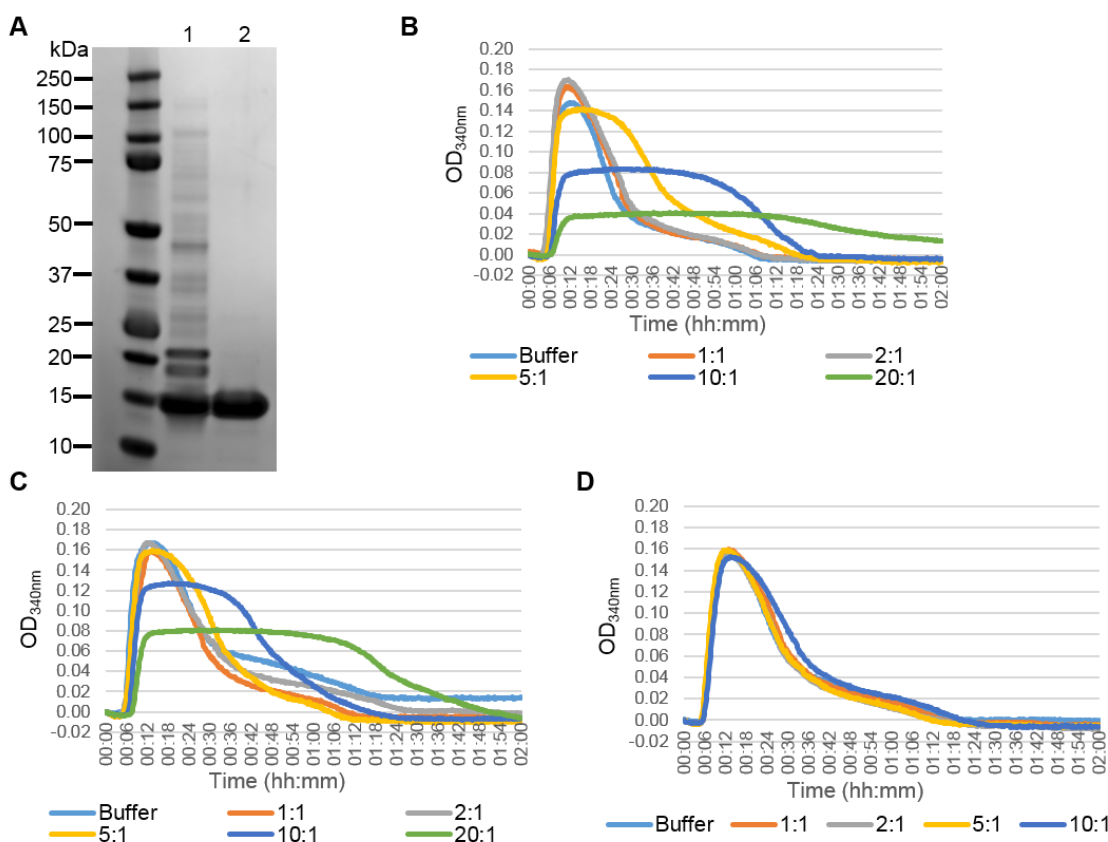


Figure 5-8 Re-growth and purification of Affimer B9. Following expression and purification of Affimer B9, size exclusion chromatography (SEC) was performed to allow the isolation of the Affimer in a monomeric form, and free from contamination. SEC fractions were collected into two different protein pools differing in purity. **A.** 20 µg of each protein pool was analysed by SDS-PAGE. Lane 1 shows the Affimer monomer protein (~13 kDa) in addition to contaminating bands and lane 2 shows the monomeric fraction. **B.C.D.** Turbidimetric assays in plasma with the addition of Affimer B9 protein at 1:1, 2:1, 5:1, 10:1, 20:1 Affimer:fibrinogen molar ratio. Buffer only (no Affimer protein) was included as a control. **B.** Turbidimetric assay in plasma using an early (active) batch of Affimer B9. **C.** Turbidimetric assay with the addition of the less pure Affimer B9 protein fraction (A., lane 1). **D.** Turbidimetric assay with the addition of the monomeric Affimer protein fraction (A., lane 2).

5.2.5 Characterising the B3-fibrinogen interaction

To define the binding of B3 to fibrinogen, plate-based ELISA assays were performed to determine the K_D of Affimer-fibrinogen interaction. Additionally, pull down assays in plasma were performed to confirm fibrinogen binding and specificity. ELISA assay showed the K_D of B3 to be 425 nM±46 nM (mean±SD) for fibrinogen (Figure 5-9A). Binding assays were also performed with fibrin, and determined that the Affimer bound with lower affinity than it did fibrinogen (872 nM±110 nM), however, binding of B3 did not reach saturation and so B3 affinity for fibrin may actually be lower than was calculated (Figure 5-9B). Pull down assays demonstrated that Affimer B3 bound to fibrinogen only weakly, even when

less stringent washing conditions were applied in the pull down protocol (lower NaCl concentrations in the wash buffers) (Figure 5-9C). The pull down assays demonstrated binding of Affimer B3 to fibrinogen, but additional bands were also present in pull down elutions when analysed by SDS-PAGE. There were 2 bands present, one at ~110 kDa and one at ~90 kDa, that appeared similar to those identified in chapter 4 as kininogen and fibrinogen γ-dimer, respectively. There was also a faint band between the 250 kDa and 150 kDa molecular weight markers that was present in the pull down elutions of both B3 and SQT His control Affimer.

The kinetics of Affimer B3 binding to fibrinogen was determined by SPR. The Affimer demonstrated fast association (K_a of 2.46±0.16 x 10^5 $M^{-1}S^{-1}$) and dissociation (K_d 2.31±0.21 x 10^{-2} S^{-1}) to fibrinogen (mean±SD).

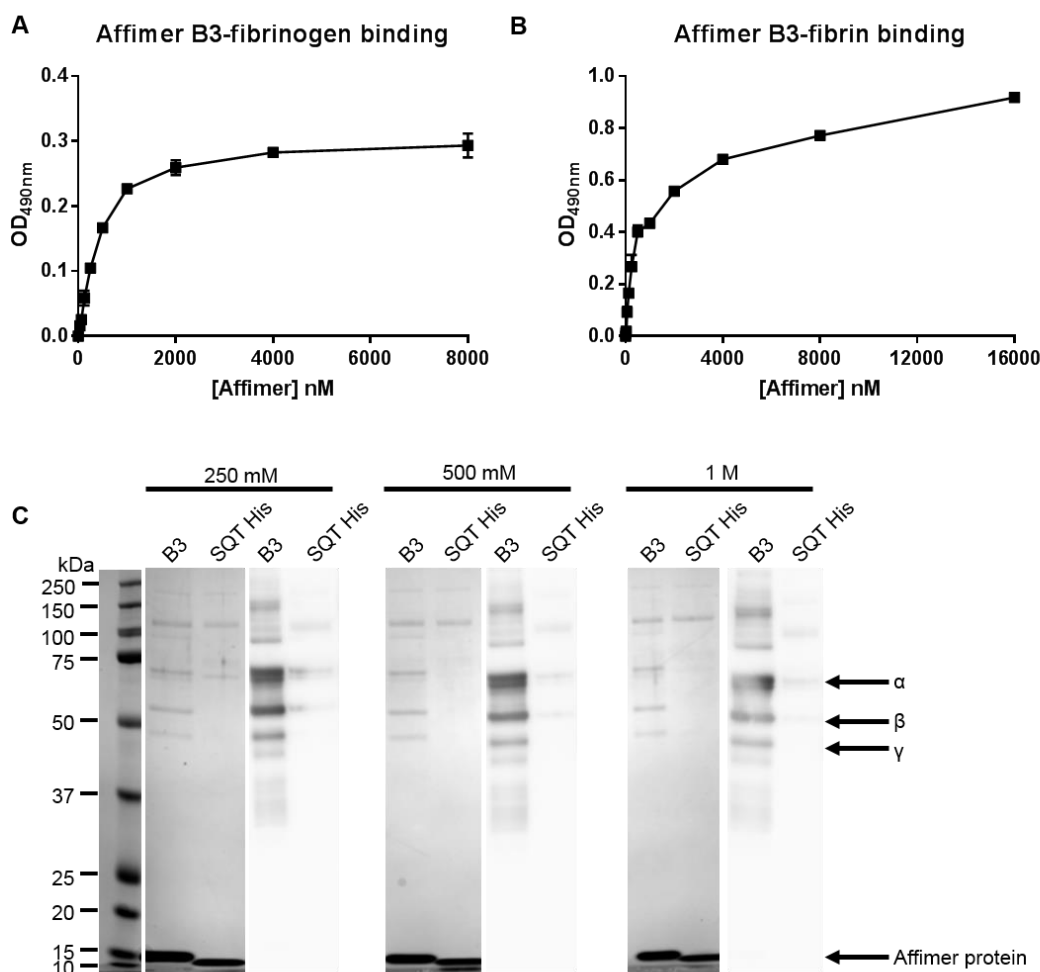


Figure 5-9 Binding characteristics of Affimer B3, determined by ELISA and pull down assay. A 96 well plate was coated with **A.** fibrinogen or **B.** fibrinogen converted to fibrin, and Affimer added to the plate in increasing concentration. After washing, Affimer was detected using an anti-polyhistidine antibody. n=3, mean±SD of single, representative experiments is shown. **C.** Pull down assays were performed with B3 and SQT His control Affimer to confirm binding of B3 to fibrinogen in solution, and determine the fibrinogen specificity of B3. B3 and SQT His were incubated with Dynabeads to allow the C-terminal his-tag of the Affimers to adhere to the Dynabead surface. Dynabeads were then incubated with plasma for 30 min prior to washing, and elution of proteins from the Dynabeads. (C) shows pull down elutions analysed by SDS-PAGE (left) and western blotting (anti-fibrinogen) (right) in pull down assays using wash buffers with increasing [NaCl] in wash buffers (250 mM, 500 mM, 1 M). The position of fibrinogen α, β, and γ chains is indicated, and position of Affimer proteins at ~13 kDa (B9) and ~10 kDa (SQT His).

5.2.6 Conformational and not linear interactions are responsible for B3 induced changes in clot structure/lysis

Linear peptides of the same sequence as the two variable regions of Affimer B3 were synthesised and modelled (Figure 5-10A-E). The linear peptides and the Affimer variable region loops were modelled by Dr Katie Simmons. The effect of the peptides on clot lysis was investigated using turbidimetric assays in pooled human plasma. In contrast to Affimer B3, the synthetic peptides did not prolong clot lysis time (Figure 5-10F).

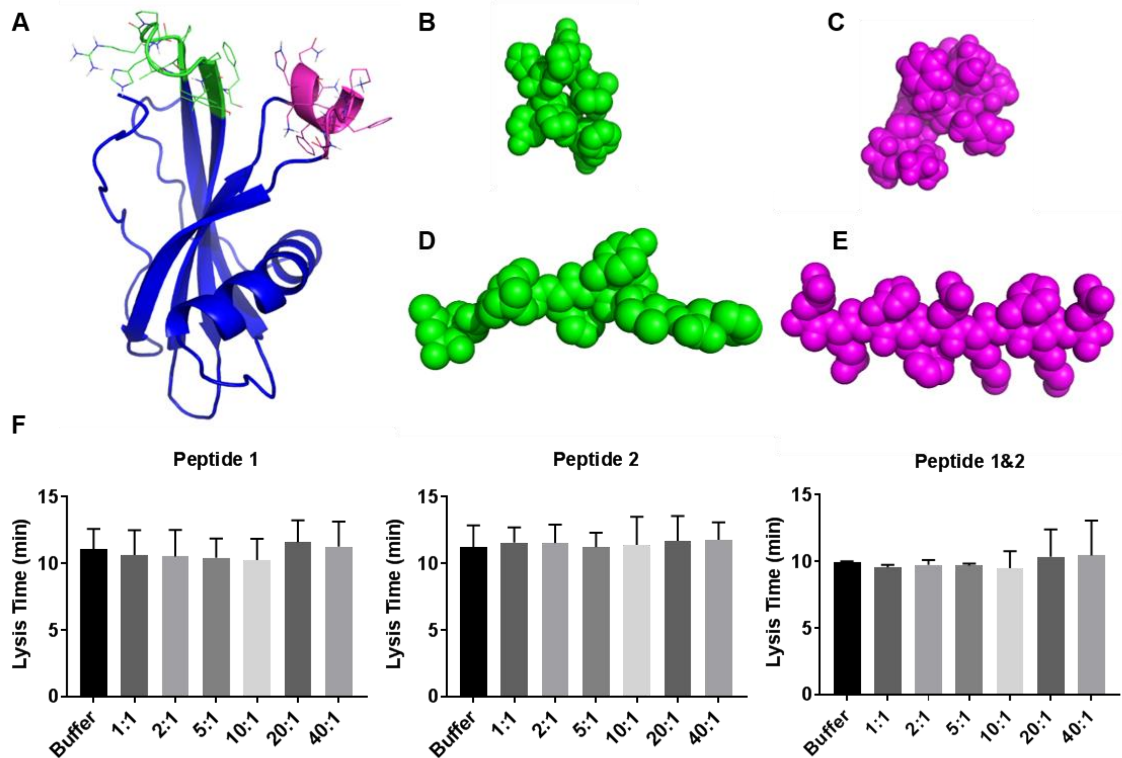


Figure 5-10 Effect of B3 variable region synthetic peptides on clot lysis in plasma. Synthetic peptides with the amino acid sequences of Affimer B3's two variable regions were synthesised, and added to pooled human plasma in a turbidity and lysis assay to assess their effect on clot lysis. Peptides were used at 1:1, 2:1, 5:1, 10:1, 20:1 and 40:1 peptide:fibrinogen molar ratio. Samples receiving buffer only (no peptide protein) were used as a control. **A.** Model of Affimer B3, based on the crystal structure of Stefin A (PDB ID 3KFQ). The Affimer scaffold is blue, variable region one is green, and variable region two is pink. **B. C.** Variable region one (B) and two (C) in the conformation these regions adopt when part of the Affimer scaffold. **D. E.** The sequences of the Affimer variable regions were also modelled as two 9 amino acid peptides using the Maestro graphical user interface, and energy minimised using the OPLS3 force-field in a water environment. **F.** Lysis time in turbidimetric assays using peptide 1, peptide 2, and peptide 1 and 2 together. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, comparisons made against buffer only control.

When comparing models of the variable region sequences, the conformation adopted by the 9 amino acid stretches were vastly different when part of the Affimer scaffold (Figure 5-10B,C) than when they were modelled as isolated short peptides (Figure 5-10D,E). These data suggest that the interaction between Affimer B3 and fibrinogen is conformational and dependent on the constraint of the variable region sequences within the Affimer scaffold.

5.2.7 The effect of B3 on clot formation/lysis in a purified fibrinogen system

B3 has thus far been shown to increase lysis time in turbidimetric assays in a plasma system, while increasing lag time and reducing maximum OD. To investigate the fibrinogen-specific effects of B3, turbidimetric assays were performed in a purified system, in which purified fibrinogen was used in place of plasma. B3 significantly increased lag time in a dose-dependent manner from 2:1 Affimer:fibrinogen molar ratio, and decreased maximum OD at all concentrations used. In contrast to its effect in plasma assays, B3 had no significant effect on lysis time in a purified system (Figure 5-11). Results are summarised in Table 5-3.

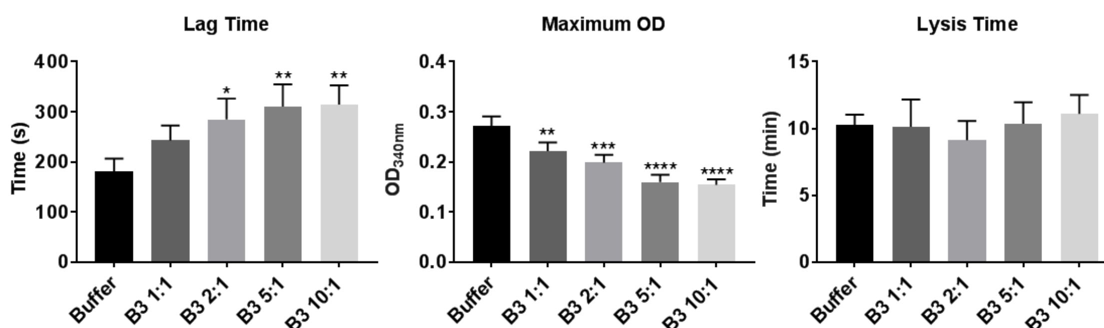
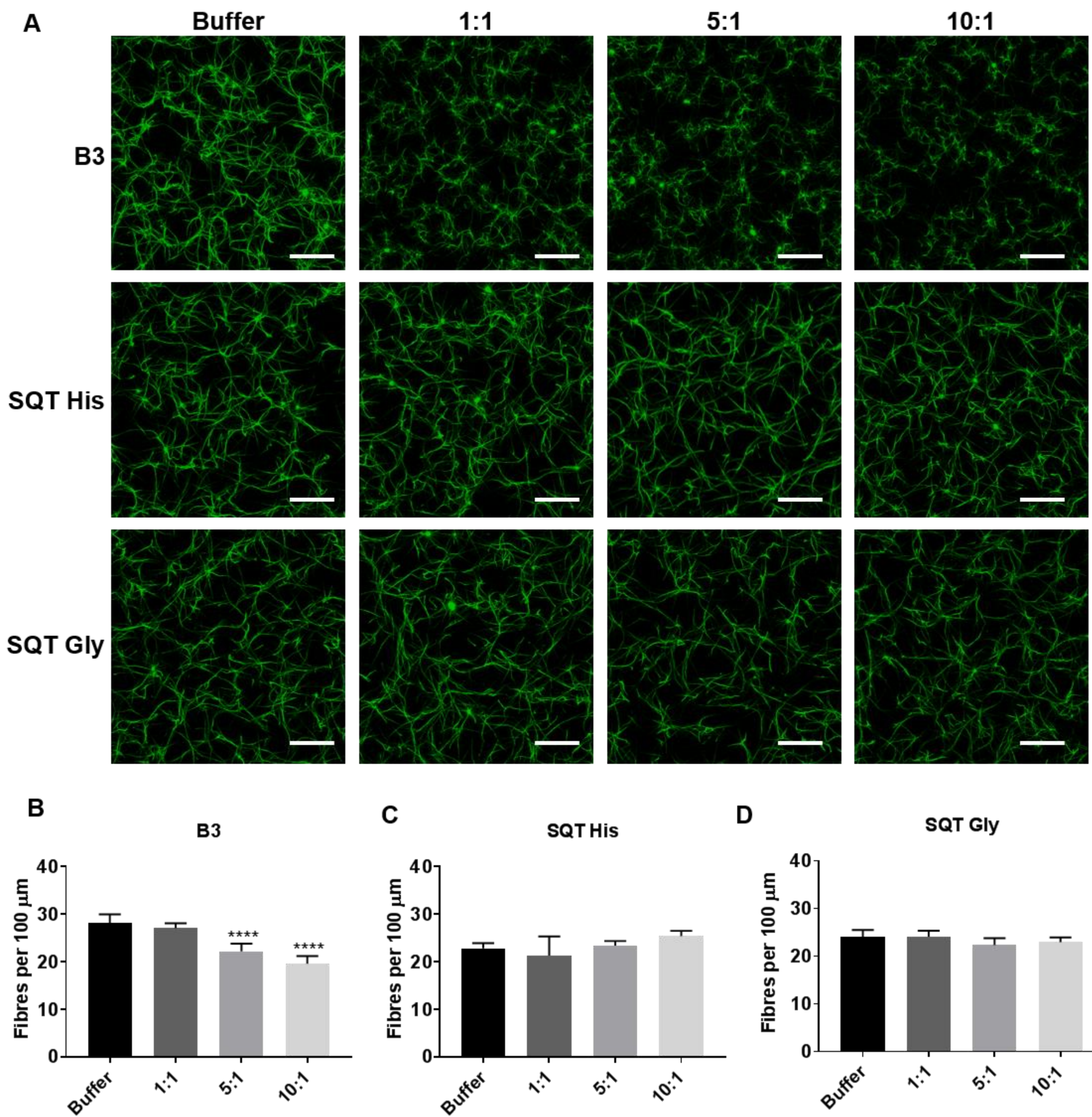


Figure 5-11 Effect of increasing doses of Affimer B3 on clot formation and lysis in a purified system. Fibrinogen was incubated with B3 at 1:1, 2:1, 5:1, 10:1 Affimer:fibrinogen molar ratio, prior to the addition of thrombin and calcium (turbidity assays) or thrombin, calcium, tPA and plasminogen (turbidity and lysis assays). Samples receiving buffer only were used as a control. Lag time, maximum OD and lysis time data shown. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 when compared with buffer only control.

Affimer: fibrinogen molar ratio	Lag time (s, mean±SD)	Maximum OD (AU, mean±SD)	Lysis time (min, mean±SD)
Buffer	180.7±26.3	0.2727±0.0181	10.3±0.8
B3 1:1	242.7±30.1	0.2218±0.0169**	10.1±2.0
B3 2:1	284.7±41.8*	0.1985±0.0157***	9.1±1.4
B3 5:1	310.7±44.5**	0.1600±0.0147****	10.4±1.6
B3 10:1	314.7±38.1**	0.1545±0.0108****	11.1±1.4

Table 5-3 The effect of Affimer B3 on clot formation and lysis parameters in turbidimetric assays in a purified system. n=3, mean±SD. Statistical analysis was performed using one-way ANOVA, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 when compared with buffer only control.

Affimer B3 did not prolong lysis time in a purified system, in contrast to the Affimer effect in plasma. The reduced maximum OD in the purified turbidity assays (Figure 5-11) indicated a change in clot structure when B3 was present. The difference in clot lysis comparing purified protein and plasma experiments is likely due to altered interaction of clots with compact structure and thin fibres (induced by B3) with plasma proteins, i.e. the compact clots generated by B3 capture more anti-fibrinolytic proteins thus reducing susceptibility to lysis. A similar interaction between clot structure and plasma proteins that resulted in different clot lysis has been described before [163]. To investigate this possibility in more detail, the effect of B3 on clot structure in a purified system was assessed (Figure 5-12A). Formation of clots with IF-1 purified fibrinogen and increasing concentrations of Affimer B3 showed that clots made with the Affimer had a reduced fibre count (Figure 5-12B), which contrasts with the effects of the Affimer in a plasma system (in which clots made with Affimer B3 were significantly more dense, Figure 5-5). In a purified system, Affimer B3 caused a small, but significant reduction in average fibre count from 28±2 fibres per 100 µm in buffer only control to 22±2 at 5:1 and 20±2 at 10:1 (mean±SD) (P≤0.0001 for both), while type I Affimer controls had no significant effect on fibre count (Figure 5-12A,C,D).



B C D

Figure 5-12 Effect of Affimers on the structure of clots made from purified fibrinogen. Representative confocal images of Affimer B3 and type 1 scaffolds in clots made from purified fibrinogen. Affimer proteins were incubated with fibrinogen at 1:1, 5:1 and 10:1 Affimer:fibrinogen molar ratio. Buffer only controls received no Affimer protein. **B.C.D.** Average fibre count of clots formed in the presence of B3, SQT His and SQT Gly. Two clots were made for each condition, and 3 images taken in different areas of each clot. Fibre density was determined by counting the number of fibres that cross 20 lines across each image, using ImageJ software. Data presented as mean±SD. Statistical analysis was performed using one-way ANOVA, ****P≤0.0001 represents difference from buffer only control. Scale bar = 20 µm.

5.2.8 Affimer B3 is not incorporated into the fibrin clot

To investigate whether Affimer B3 was functioning via incorporation into fibrin clots, the Affimer was labelled using Alexa-488 dye (green) and plasma clots formed with Alexa-594 (red) labelled fibrinogen were viewed by confocal microscopy. The incorporation of Affimer B3 was compared with that of Affimer B9, in both monomeric and multimeric form (various forms of Affimer B9 have been discussed previously, in section 5.2.4). Incorporation into clots was determined by counting the number of yellow pixels in merged images, yellow pixels arose when Affimer (green) and fibrinogen (red) signal overlapped. Figure 5-13 shows that, when compared with SQT Gly scaffold control, B3 was not significantly incorporated into the clot. In comparison, Affimer B9 was significantly associated with fibrin fibres, when the Affimer was in a monomeric and a multimeric form, the latter showing most incorporation into clots (Figure 5-13B).

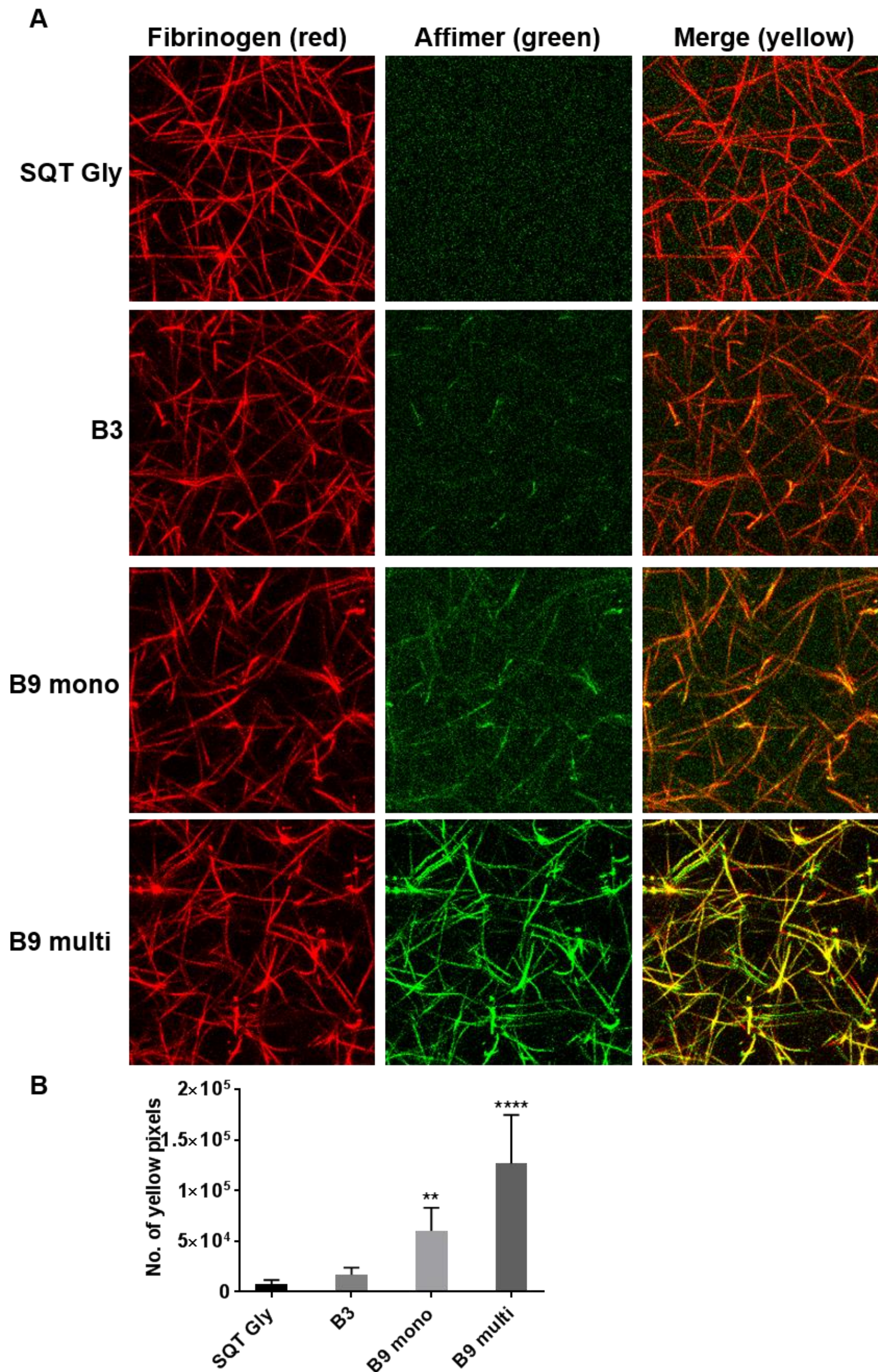


Figure 5-13 Incorporation of fluorescently labelled Affimer proteins into fibrin clots. A. Representative confocal images of plasma clots incubated with either Alexa-488 labelled Affimer B3, B9 (monomeric or multimeric form), or SQT Gly scaffold control at 5:1 Affimer:fibrinogen molar ratio. Plasma also included the addition of Alexa-594 labelled fibrinogen. **B.** Number of yellow pixels in merged images, quantified by ImageJ Colour Pixel Counter plugin. Two clots were made for each condition, and 3 images taken in different areas of each clot. Data presented as mean±SD, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$ when compared with SQT Gly control using one-way ANOVA.

5.2.9 B3 must be present during clot formation to have an effect on the lysis of clots

To further investigate B3 mechanisms of action, turbidimetric assays were performed in which the Affimer was added either at the start of the experiment (and so was present during clot formation), or added to the top of a pre-formed clot. The results demonstrated that when B3 was added to plasma prior to initiating clot formation, the Affimer caused a reduction in maximum OD (Figure 5-14A) and drastically reduced lysis after 300 min compared to buffer only control. The clot formed in the presence of buffer reached 100% lysis by the 300 min time point, whereas the sample made in the presence of B3 had only reached 11.2±1.0 % ($P \leq 0.0001$) lysis (mean±SD) (Figure 5-14B). In contrast, when B3 was added on top of a formed clot it was unable to cause a prolongation in lysis (Figure 5-14C,D). These data suggest that B3 exerts an anti-fibrinolytic effect primarily through changes during the process of clot formation, including changes to clot structure.

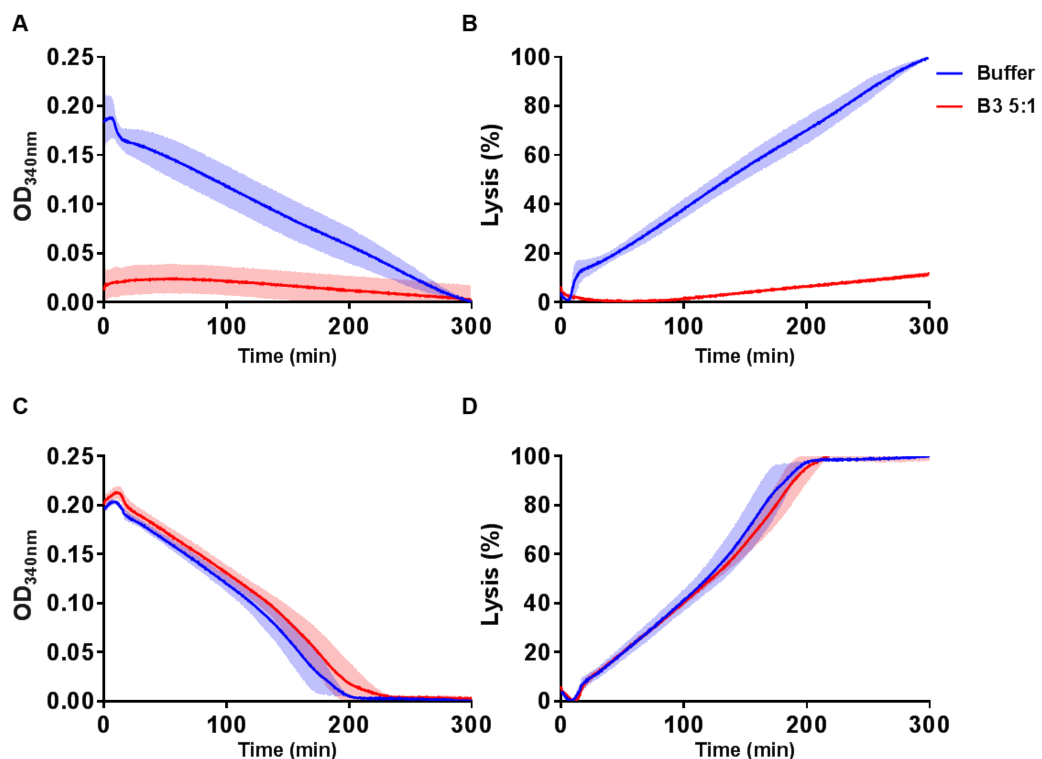


Figure 5-14 The lysis of fully formed clots formed in the presence and absence of Affimer B3. A. Pooled plasma with either B3 at 5:1 Affimer: fibrinogen molar ratio, or buffer was clotted with thrombin and calcium and left to reach maximum turbidity for 1 h. After 1 h, tPA was added, turbidimetric data shown. **B.** Clot lysis (%) over time of clots in (A). **C.** Pooled human plasma was clotted with thrombin and calcium, and left for 1 h to reach maximum turbidity. After 1 h, Affimer B3 or buffer was added to the clot immediately before the addition of tPA. (C) shows turbidimetric data after tPA and Affimer added. **D.** Clot lysis (%) over time of clots in (C). n=3, mean±SD.

5.2.10 Identification of a potential site of B3-fibrinogen interaction

To investigate possible sites of interaction between Affimer B3 and fibrinogen a pull down assay was performed. The Affimer was pre-incubated with fibrinogen to enable formation of Affimer/fibrinogen complexes. Plasmin was added to digest these complexes and generate fibrinogen degradation products (FDPs). The Affimer/fibrinogen complex was digested for between 20 min and 2 h. The rationale behind digesting for different times with plasmin was to enable isolation of the smallest Affimer-binding FDP. The Affimer-binding FDP was then identified by mass spectrometry, in combination with comparison with published molecular weights of FDPs.

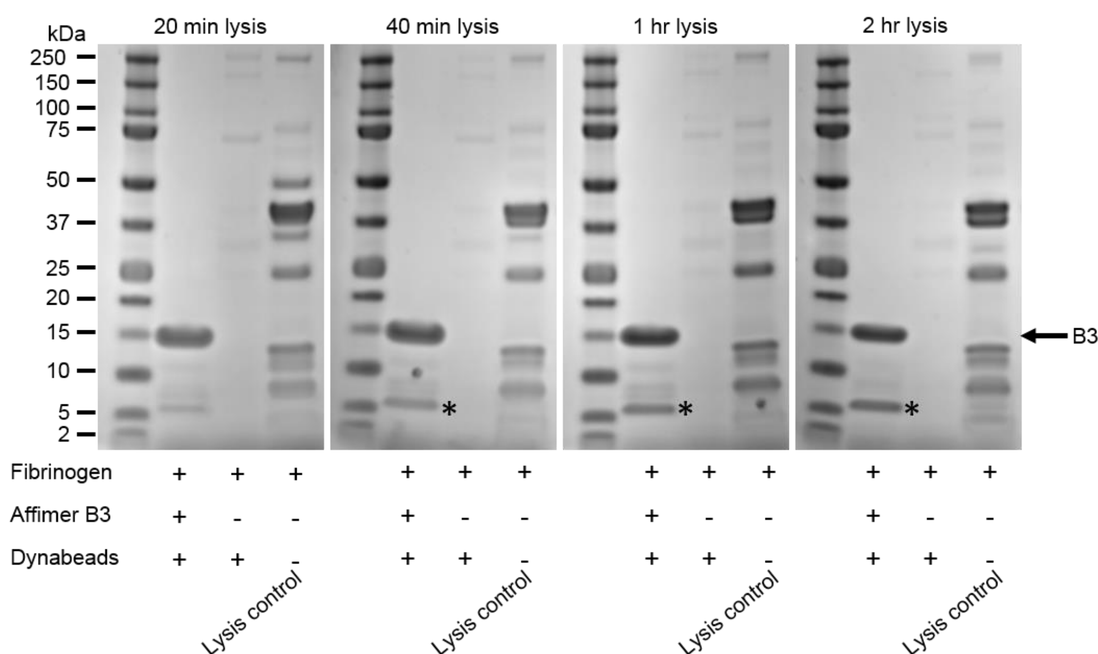


Figure 5-15 Pull down assay to investigate Affimer B3-fibrinogen interaction. A pull down was performed in which fibrinogen was incubated with Affimer B3, prior to the addition of plasmin. Lysis was allowed to proceed for either 20 min, 40 min, 1 h or 2 h, before aprotinin was added to clots to prevent further digestion. Affimer-interacting protein complexes were isolated with affinity beads and pull down elutions analysed by SDS-PAGE. Controls containing no Affimer were run to assess binding of fibrinogen degradation products (FDPs) to the Dynabeads in the absence of Affimer. "Lysis controls" were included to look at the whole population of FDPs generated during reactions. The proteins marked with * were identified by mass spectrometry (LC-MS/MS).

Protein elutions containing Affimer-binding FDPs were analysed by SDS-PAGE (Figure 5-15). Affimer B3 pulled out a fibrinogen fragment at all lysis time points which ran at ~6 kDa that was identified by mass spectrometry as containing primarily fibrinogen β chain. Figure 5-15 shows that the FDP pulled out by B3 was

not present in the lysis controls, indicating that the presence of the Affimer during plasmin digestion of fibrinogen yielded a different population of FDPs and thus may have been directly blocking plasmin cleavage site(s). To confirm this observation, an experiment was performed to allow the comparison of FDPs formed in the presence and absence of Affimer B3 (Figure 5-16A).

The gels in Figure 5-16A confirmed that the presence of Affimer B3 during plasmin digestion of fibrinogen yielded additional FDPs than when fibrinogen was lysed without the Affimer present. In particular, the presence of protein bands at ~6 kDa, and one at ~4 kDa (Figure 5-16A, white arrows). Based on these observations, a fibrinogen binding site for Affimer B3 at the N-terminus of the β chain was proposed. Figure 5-16B shows the location of plasmin cleavage sites on the fibrinogen β chain [361]. Cleavage at these sites would yield FDPs of molecular weight 2374 Da, 2236 Da and 1112 Da (Figure 5-16C). Blockage of plasmin cleavage sites K22 and R43 by B3 while allowing cleavage at K54 would result in the release of an FDP of ~6 kDa (Figure 5-16D), as was observed in Figure 5-15 in B3 pull down elutions and B3-containing samples in Figure 5-16A. The presence of a protein band at ~4 kDa in sample containing B3 after 1 h lysis suggests that cleavage at R43 can occur in the presence of B3 after prolonged plasmin digestion (Figure 5-16A).

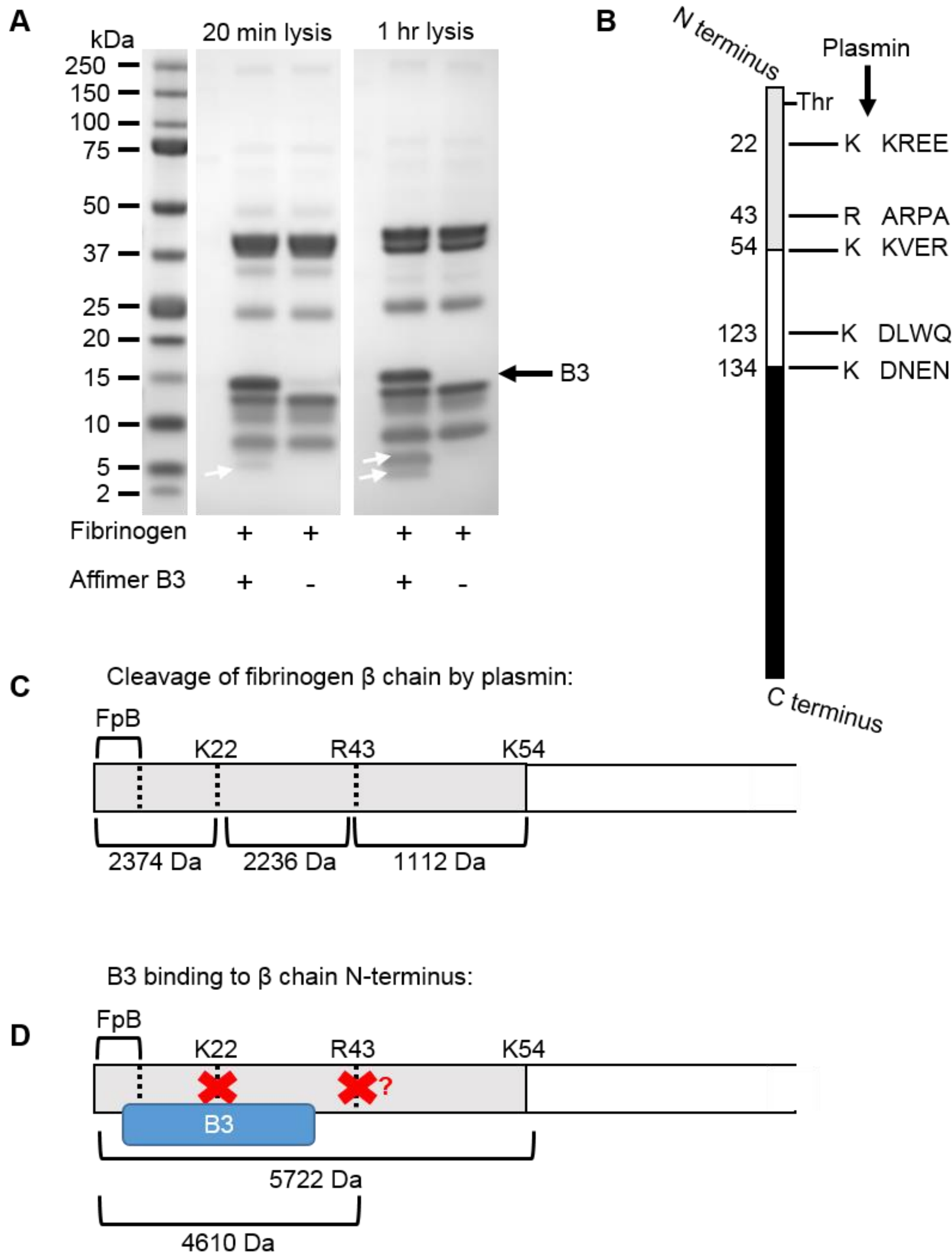


Figure 5-16 Investigation of potential Affimer B3-fibrinogen interaction site on the fibrinogen β chain. A. Fibrinogen was incubated with or without Affimer B3, prior to plasmin digestion for the time indicated above each gel. White arrows indicate FDPs at 6 kDa and 4 kDa present only with Affimer B3. **B.** Plasmin cleavage sites on human fibrinogen β chain. The N-terminus is grey, fragment E is white, and D black. The numbers indicate the positions of the new N-terminal residues after plasmin cleavage. The thrombin (thr) cleavage site is indicated. Image adapted from Zwaal and Hemker (1986). **C.** N-terminus of the β chain with the molecular weight of the products of plasmin digestion at each cleavage site. **D.** Hypothesised binding site of B3 on fibrinogen β chain. Blocking of the indicated plasmin cleavage sites (red crosses) would yield degradation products of 4610 Da and 5722 Da.

5.2.11 Affimer B3 does not prolong lysis time in whole blood

Experiments in whole blood were performed to determine if the presence of blood cells influenced the anti-fibrinolytic properties of Affimer B3. EXTEM experiments with tPA (lysis experiments) (Figure 5-17A-D) and without tPA (Figure 5-17E-H) were used to investigate the effect of increasing concentrations of Affimer B3 on clot lysis and clot formation in whole blood. Experiments with fib-tem reagent allowed investigation of Affimer effects on the fibrin component of the clot, without the influence of platelets (Figure 5-17I-K). Figure 5-17A and Figure 5-17D show that the Affimer did not delay lysis in whole blood. In EXTEM experiments, the blood samples receiving Affimer B3 actually underwent a greater degree of lysis than clots without Affimer. Samples receiving buffer only underwent on average 13.3±4.5% (mean±SD) clot lysis over the duration of the EXTEM experiments, while clots receiving B3 had lysed 28.0±8.7% ($P \leq 0.05$) (Figure 5-17H). In terms of the effects of the Affimer on clot strength, the presence of B3 caused a significant reduction in maximum clot firmness in lysis experiments (Figure 5-17C), EXTEM (Figure 5-17G) and FIBTEM (Figure 5-17K) experiments. Clotting time was delayed in the presence of B3 in all three types of experiment performed, which was only significant in lysis experiments (Figure 5-17B). The presence of Affimer B3 delayed clotting time from 74.3±8.5 s (mean±SD) to 123.3±31.6 s with B3 at 10:1 Affimer:fibrinogen molar ratio ($P \leq 0.05$) (Figure 5-17B).

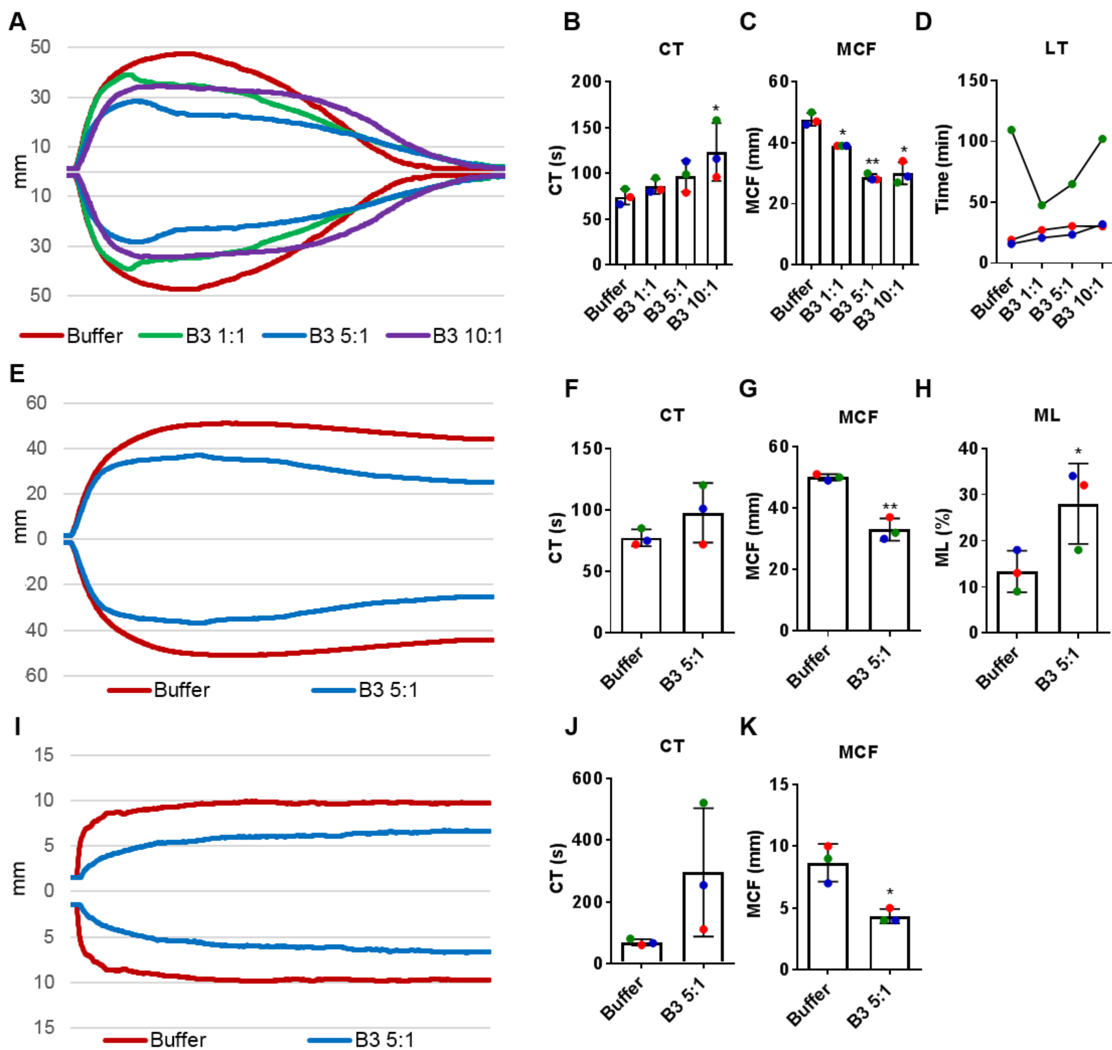


Figure 5-17 The effect of Affimer B3 on clot formation and lysis in whole blood ROTEM. Whole blood was added to ROTEM cups and clotting initiated with ex-tem reagent (containing tissue factor). Affimer was added to the whole blood to final concentrations of 1:1, 5:1, 10:1 Affimer:fibrinogen molar ratio in lysis experiments (A), and 5:1 in EXTEM (E) and FIBTEM (I) experiments. Buffer only was included as a control. **A-D.** Lysis experiments were performed by adding tPA to the reaction mixture to allow clot lysis to proceed. **A.** Representative trace. **B.** Clotting time (CT) calculated from lysis experiments. **C.** Maximum clot firmness (MCF) calculated from lysis experiments. **D.** Lysis time (LT), calculated as time to 50% clot lysis. **E-H.** EXTEM experiments were also performed without tPA, to look at the effect of the Affimer on clot formation without concurrent lysis **E.** Representative trace. **F.** CT of samples in EXTEM experiments. **G.** MCF in EXTEM experiments. **H.** Maximum lysis (ML), calculated as the difference between MCF and the lowest amplitude after MCF, described as a percentage of MCF. **I-K.** Experiments were performed with fib-tem reagent, which contains platelet inhibitor cytochalasin D, to allow the effect of the Affimer on the fibrinogen component of the blood clot to be assessed. **I.** Representative trace. **J.** CT **K.** MCF. n=3, each individual's data is presented in a different colour (red, green, and blue) in B-D, F-H, and J-K to show inter-individual variability. ROTEM traces in A, E, I are from the same individual. B-D, F-H, J-K is presented as mean±SD. Statistical analysis was performed using one way ANOVA (B-D) or two-tailed paired student *t*-test (F-H, J-K). *P≤0.05, **P≤0.01 when compared with buffer only control.

5.3 Discussion

This chapter describes the characterisation of type I fibrinogen-binding Affimers following a screen by Avacta. Of the six type I fibrinogen binders isolated, two Affimers, B3 and B9 had an effect on clot lysis following initial screens for Affimer effects in plasma turbidimetric assays. The two Affimers had some sequence homology, suggesting that they may interact with the same region of fibrinogen, providing one possible explanation for the similarities in the effects of the two Affimers. The presence of both Affimers caused a prolongation in lag time in a plasma system, and reduction in maximum OD, with B3 displaying a greater effect on both of these parameters at lower concentrations than were required for B9. Although Affimer B3 caused a significant reduction in maximum OD, clot formation was not prevented by this Affimer, as presence of fibrin fibres was confirmed with confocal and SEM. The reduction in maximum OD by B3 and B9 suggested changes to fibrin clot structure, which was visualised by confocal microscopy with plasma clots formed in the presence of increasing concentrations of the Affimers. Both B3 and B9 caused the formation of more dense clot structures, confirmed with permeation experiments which showed a reduction in porosity of plasma clots. The formation of more dense clots was not evident in SEM images, however, these did show that the presence of B3 and B9 in plasma clots was associated with formation of thinner fibrin fibres. More dense clots with thinner fibres are typical of fibrin networks with enhanced resistance to lysis [82]. The differences in clot architecture as viewed by confocal or SEM are likely due to differences in the sample preparation required for the two methods. Confocal experiments are performed with hydrated clots and are therefore more likely to represent the clot structure under physiological conditions. Dehydration of clots during SEM preparation may disrupt clot structure, but this method is important because it enables investigation of the characteristics of individual fibrin fibres.

Pull down assays in plasma confirmed the ability of B3 and B9 to bind to fibrinogen in solution. Type I scaffold control SQT His showed no binding to fibrinogen, as expected from the unreactive nature of this Affimer in assays performed thus far. Affimer B9 pull down elutions contained multiple bands in addition to fibrinogen, which were identified as containing Affimer protein

aggregates in addition to *E. coli* proteins which are likely to have originated from the bacterial system used to express the proteins.

Formation of Affimer protein aggregates was discussed in chapter 3. The propensity for Affimers to aggregate has been reported by other groups and is not necessarily a reason for dismissing an Affimer from investigation. In fact, others have reported that some Affimers have a higher affinity for their target when they are in a dimeric form [298]. However, upon SEC by Avacta to purify the Affimer to a higher degree, the monomeric Affimer fraction displayed none of the Affimer's previously seen activity in turbidimetric assays. Additionally, the Affimer B9 fraction of a lower purity, when analysed by SDS-PAGE, contained proteins with molecular weights not consistent with expected weights of Affimer aggregates, which may again have been due to contamination with bacterial proteins. It is possible that the Affimer forms more functionally active multimers which subsequently have a high affinity for bacterial proteins, and the two are inseparable by SEC. It is also possible that it is the Affimer-binding bacterial proteins in the B9 multimeric fractions that contribute to the activity of the Affimer in plasma. Interestingly, the monomeric form of B9 showed less incorporation into fibrin clots than the multimeric form of the Affimer, as determined by confocal microscopy. Many bacterial proteins are known to bind to fibrinogen [362], and it may be fibrinogen-binding bacterial proteins contributing to this elevated incorporation of multimeric B9. B9 was excluded from further study upon determining that functional Affimer protein could not be separated from the large number of contaminant protein species.

Having excluded B9 from further investigation, pull down assays with Affimer B3 were performed. These assays were performed with a range of salt concentrations in the wash buffers (previously, 1M NaCl had been used) to provide less stringent wash conditions, due to Affimer B3's relatively low affinity for fibrinogen as determined by ELISA (~400 nM). These assays confirmed an ability of the Affimer to bind to fibrinogen in solution but suggested a transient binding interaction between the Affimer and fibrinogen, due to low yield of fibrinogen in B3 pull down elutions. Affimer B3 pull down elutions also contained proteins that weren't fibrinogen α, β or γ chain. There was a protein band at ~110 kDa that appeared similar to that identified in chapter 4 as kininogen. As

discussed in chapter 4, this protein was likely non-specifically binding to the His-tag affinity beads. There was also a faint protein band between the 150 and the 250 kDa molecular weight markers, but this was also present in the SQT His control and not specifically binding to Affimer B3. If work with Affimer B3 was to continue, it would be of value to identify this protein band as a potential binder to the type I Affimer scaffold backbone.

Given the Affimer's limited ability to pull fibrinogen from plasma in pull down assays, the kinetics of the Affimer-fibrinogen interaction were investigated in more detail using SPR. SPR showed the Affimer to have a fast association and dissociation rate from fibrinogen, which could explain the inability to stably bind fibrinogen and subsequently pull it down in pull down assays. However, Affimer F5 (described in chapter 4) had a similarly fast dissociation rate and was able to pull down relatively large quantities of fibrinogen from plasma under similar protocol conditions. Further investigation would be required to understand the Affimer B3 pull down results, which would include repeating these assays and monitoring the wash buffers for fibrinogen content. This would show if Affimer B3 was binding fibrinogen which was subsequently washed off, or if the Affimer had less fibrinogen-binding capacity from the outset of the experiment.

In plate based ELISA assays Affimer B3 showed a higher affinity for fibrinogen than fibrin, suggesting that the Affimer was binding to a region of fibrinogen that undergoes conformational change once converted to fibrin. This finding, in combination with others (discussed below) indicated that the Affimer acted through binding/interference with fibrinopeptide B (FpB) and/or structures involved in lateral aggregation of protofibrils.

If the hypothesis of B3-FpB binding were correct, then one would expect Affimer B3 to show no binding to fibrin (due to a lack of FpB), however, this assumes that addition of thrombin to immobilised fibrinogen in the ELISA assay allowed conversion of the entire population of fibrinogen molecules to fibrin. The approximately two-times lower affinity of the Affimer for fibrin than for fibrinogen could be explained if only about 50% of the fibrinogen molecules were converted to fibrin following thrombin cleavage of fibrinopeptides, or that some of the fibrinopeptides remain within the surface-bound fibrinogen structure on the ELISA

plate surface. In support of this, others have shown that adsorption of fibrinogen to a surface results in reduced accessibility of the E region to thrombin, and reduced release of FpB from surface-adsorbed fibrinogen [363]. Work by Riedel et al. (2011) demonstrated that thrombin cleaved approximately 64% or 35% of FpB from surface adbsorbed fibrinogen when fibrinogen was used to coat plates at high or low concentration, respectively [363].

Cleavage of FpB by thrombin is not necessary for fibrin polymerisation, but does have a role in enhancing lateral aggregation into thicker fibres [21, 364]. At least part of the effect of FpB cleavage on lateral aggregation is a result of the release of αC domains, which become available for intermolecular interactions between protofibrils [334]. Batroxobin, an enzyme derived from snake venom selectively cleaves FpA from fibrinogen, without cleaving FpB, resulting in desA fibrin monomer [365]. Clots formed with desA fibrin have thinner fibres and fewer pores than clots formed in the presence of thrombin, which cleaves both fibrinopeptides [366-368]. Clots formed from desA fibrin also display longer lag times [367]. The characteristics of clots formed following FpA but not FpB release are similar to those when clots are formed with Affimer B3. However, it has been reported that clots produced with batroxobin are less stable with respect to plasmin cleavage than clots formed with thrombin [21, 369], which is contrary to the prolongation of fibrinolysis seen in plasma clots with the addition of Affimer B3, but consistent with the inability of the Affimer to prolong lysis in whole blood. In whole blood ROTEM, the presence of Affimer B3 caused enhanced lysis of clots.

In a purified system, Affimer B3 prolonged lag time and reduced maximum OD, as in a plasma system, but had no effect on lysis time. Confocal microscopy experiments showed that clots formed with B3 had altered fibrin structure in a purified system. Although fibre counting determined a significantly lower fibre count in the presence of the Affimer in purified fibrinogen clots, visual inspection of clot images suggested formation of fibres which were shorter and finer and unequally dispersed, such that some areas of the clot appeared devoid of fibres, while others contained many fibres. This finding reflects an inability of the ImageJ macro used to count fibres to distinguish such subtleties in fibrin characteristics. However, the effect of B3 on clot structure in a purified system was still in contrast to the effect of the Affimer in a plasma system, which showed formation of vastly

more dense clots in the presence of the Affimer, a finding confirmed by permeation experiments using plasma clots. It is possible that in a plasma system, quantity of crosslinked anti-fibrinolytic proteins into the clot is altered by Affimer B3-induced change in fibrin structure. It may be that the composition of these fibrin-associated proteins is different quantitatively or qualitatively in the presence of the Affimer, thereby compromising fibrinolysis. Such a relationship between findings in purified and plasma systems has been reported by others [163]. This hypothesis is consistent with the finding that the Affimer must be present during clot formation (and thus alter fibrin clot structure) to exert its anti-fibrinolytic effect.

The formation of clots made up of thin fibres in the presence of Affimer B3 is strong evidence for an interaction of the Affimer with the molecular contacts involved in protofibril formation and/or lateral aggregation of protofibrils. The Affimer may interfere with B:b knob:hole interactions or the αC intermolecular contacts between protofibrils [18]. The requirement for the Affimer to be present during clot formation to prolong fibrinolysis also indicated an involvement in these initial steps of clot formation that influence final clot structure. Confocal microscopy experiments utilising labelled Affimer B3 showed minimal incorporation of the Affimer in the fibrin clot, again suggesting binding to a region of fibrinogen which is lost or altered in the final fibrin clot structure. Discussion of a possible effect of the Affimer on FpB release has been provided above. In addition to FpB cleavage, there is release of αC regions upon the conversion of fibrinogen to fibrin [334], which is involved in interactions between protofibrils and formation of thick fibrin fibres. Studies using mutant forms of fibrinogen lacking the C-terminal portion of the α chain have demonstrated that preventing αC domain interactions results in longer lag times and formation of fibrin clots made up of thinner fibres which were more densely packed and less permeable [86, 370]. Collet et al. (2005) found lysis of α251 clots to be enhanced in purified systems [370], contrary to what is seen with Affimer B3 in a similar system (Affimer B3 had no effect on lysis in a purified system), but consistent with the Affimer effect in whole blood.

In order to investigate potential Affimer-fibrinogen interaction sites, a pull down was performed in which Affimer B3 was incubated with fibrinogen prior to plasmin

digestion, and isolation of Affimer-fibrinogen degradation product (FDP) complexes. When pull down elutions were analysed by SDS-PAGE, there was a protein band at ~6 kDa pulled down by Affimer B3 present at all lysis time points. These 6 kDa bands were not present in lysis controls displaying the entire population of FDPs generated by plasmin digestion of fibrinogen in the absence of the Affimer. A subsequent experiment was performed to allow direct comparison of FDPs generated with and without the Affimer present. Plasmin digestion of fibrinogen in the presence of Affimer B3 yielded FDPs of 4 kDa and 6 kDa not present in fibrinogen digested without the Affimer and indicated that B3 was directly blocking plasmin cleavage of fibrinogen to give these additional FDPs. The B3-binding ~6 kDa protein pulled down by the Affimer was sent for identification by mass spectrometry (MS) and contained primarily fibrinogen β chain. However, MS results reported a large number of non-fibrinogen contaminant proteins. A possible explanation for the low signal to noise ratio from MS analysis is that the small size of the protein to be identified meant that it contained few trypsin digestion sites, and thus did not yield a large number of tryptic fragments for detection and identification by MS.

Based on the finding that B3 appeared to be pulling down a portion of the β chain, and blocking plasmin digestion to yield FDPs of roughly 6 kDa and 4 kDa, a binding site at the N-terminus of the β chain was proposed. Binding at this site by the Affimer would potentially limit cleavage of FpB by thrombin, and thus affect release of the αC regions. As described above, changes of this nature have been associated with altered clot structure and fibrinolytic properties consistent with the effects of Affimer B3. Binding of the Affimer to this region of fibrinogen cannot be explored with molecular modelling simulations as has been used previously (chapter 4) due to there being no published crystal structure of the β chain N-termini, or the αC regions. Although Affimer B3 is interesting in how it functions mechanistically, the inability of the Affimer to prolong lysis in whole blood is justification to halt further investigation and characterisation of the Affimer.

ROTEM studies demonstrated that Affimer B3 did not prolong lysis in whole blood as it did in plasma. In fact, the Affimer enhanced the lysis of whole blood clots when compared to buffer only control, while the delays in clotting time were consistent with prolonged lag phases in turbidimetric experiments. Reduction in

maximum clot firmness (MCF) reflects a reduced mechanical strength of the clot formed in the presence of the Affimer, and is mainly determined by fibrin concentration, fibrin polymerisation, FXIII activity, but also contributions from platelet function [322]. FIBTEM experiments confirmed that the reduction in MCF was, at least in part, due to an effect on the fibrin component of the clot. FIBTEM assays are initiated with tissue factor as in the EXTEM assay, with the addition of platelet inhibitor cytochalasin D, which blocks platelet activation, cytoskeletal reorganisation, and expression and activation of GPIIb/IIIa (fibrinogen) receptors. Therefore, platelet contribution to clot formation and clot strength is not a factor in this assay, and these parameters are defined only by the fibrin(ogen) component of the sample [322]. The formation of a weaker clot is likely due to the formation of thinner fibres in the presence of the Affimer, although an effect on platelet-fibrinogen interaction cannot be ruled out.

Although disappointing that the Affimer showed no prolongation of lysis in whole blood, these ROTEM findings have informed future work. Future investigation and characterisation of Affimers should include whole blood studies soon after their isolation, given that the Affimer effects in plasma turbidimetric assays are not necessarily consistent in whole blood. If Affimers are to be characterised for the purpose of providing therapeutically relevant insight into matters of fibrin clot stability, they must show function in whole blood systems.

Other aspects of the characterisation of Affimer B3 have provided useful insight for future Affimer studies. The effect of Affimer B3 on fibrin clot structure, and the suggestion that it functions through a mechanism quite dependent on changes to clot structure is something that may be best avoided in the future. There are numerous reports on the associations between clot structure and lysis time [82, 132, 137], however, modulation of the fibrin network and alteration of fibrin fibre characteristics can produce clots which are (in the case of Affimer B3) weak due to thinner fibre formation. Additionally, clots made up of thin fibres and small pores are associated with longer lysis times, but also with thrombotic complications [358, 371, 372]. It may be that an alternative approach with a more neutral effect on fibrin clot structure would be beneficial. For example, isolation of Affimers which prevent binding of lytic enzymes plasminogen and/or tPA to the

clot, thus providing an anti-fibrinolytic mechanism that need not be dependent on changes to fibrin clot structure.

An additional alternative approach would be isolation of a fibrin (vs fibrinogen) specific Affimer. A fibrin-specific Affimer would be targeted to the site of injury and not fibrinogen systemically and therefore may be more relevant in identification of therapeutically valuable targets in bleeding. Another group has had success in developing a haemostatic agent to minimise bleeding following trauma, which specifically targets fibrin. This FXIII mimetic has shown promise in a rat bleeding model [279]. The synthetic polymer showed an ability to be administered systemically but accumulate locally at sites of vascular injury and restore haemostasis due to its fibrin-binding, without exerting an effect on fibrinogen [279]. The fibrin network is also a target in fibrin sealant application (as discussed in chapter 4). In contrast to trauma, a literature search yielded no evidence for other groups utilising a fibrin-targeted strategy in the development of potential therapeutics for bleeding in haemophilia. Non-clotting factor based therapies for haemophilia include anti-TFPI antibody and si-RNA to reduce antithrombin levels, both aimed at restoring haemostasis through bypassing deficient coagulation proteins [373]. These therapies would likely have an effect on stabilising the fibrin clot through indirect effects on coagulation factors, but potentially carry a lower risk of adverse changes in clot structure.

To summarise, this chapter describes the isolation of type I fibrinogen-binding Affimers and characterisation of Affimer B3. Affimer B3 was found to cause prolongation of lysis time in plasma but not purified or whole blood systems. The Affimer appeared to function primarily through changes to fibrin clot structure, causing the formation of clots with thinner fibres and smaller pores. This change to clot structure was hypothesised to be due to interruption of B:b knob:hole interactions or limiting intermolecular αC interactions required for lateral aggregation of protofibrils. The inability to function in a whole blood system limits the potential for therapeutic development of this Affimer but this study has nonetheless provided useful insight which should influence future Affimer studies.

Future work should include isolating larger number of fibrinogen-specific type I Affimers by targeting earlier rounds of panning. This will ensure having Affimers

with varying degrees of affinity that may have the potential for clinical use. Moreover, further screening is required to identify fibrin-specific Affimers that would target blood clots that are already formed, thus reducing the risk of widespread clot formation when these molecules are applied in clinical studies.

Chapter 6 Use of Affimers for protein purification and visualisation of fibrin networks

6.1 Introduction

Thus far, Affimer proteins have been investigated for their functional effects on clot formation and clot lysis. It was also of interest to determine if the fibrinogen-binding Affimers isolated during panning were able to perform additional functions as reagents. Therefore, I investigated the use of Affimers for fibrinogen purification and also for imaging of fibrin structure in confocal microscopy.

Commercially available human fibrinogen has been shown to contain numerous impurities including α2-macroglobulin, fibulin-1, complement C3, albumin [374], and FXIII [335]. A commonly used method for further purifying commercially available fibrinogen preparations is affinity chromatography using IF-1 monoclonal antibody [317] (Kamiya Biomedical). However, this antibody is no longer produced, and thus there is a need to develop new means of obtaining highly pure fibrinogen. Affimer proteins are ideal candidates for use in affinity purification because of the ability to isolate Affimers that are highly specific for the target protein, and which can be produced in large quantities in a bacterial system. Additionally, the stability of the Affimer scaffold means these proteins are able to withstand the harsh chemical conditions often present in affinity purification protocols during wash, elution and column regeneration steps [289, 297]. Others have described an alternative method for fibrinogen purification using a fibrinogen-binding bacterial protein, which has well defined interactions with fibrinogen [375]. Although this method purified a large quantity of functional fibrinogen, there was contamination of eluted protein with bacterial fibrinogen-binding protein (1 ng in 5 μg) [376]. To my knowledge, there are no current methods of acquiring highly pure fibrinogen from plasma samples, and thus Affimers may offer a tool to purify this protein free of contaminants commonly found in commercial preparations.

Use of engineered protein scaffolds in imaging applications has been described by others [290]. Specifically, the use of Affimers for confocal microscopy imaging has been described, in which Affimers were directly labelled with fluorescent dye molecules or labelling was site-directed via a modified Affimer structure [297]. Again, the ability to isolate Affimers against a specific target, their small size and ease of production are all attractive qualities for a protein in this application. The

use of a fluorescently labelled fibrinogen-binding Affimer for visualisation of clot structure is described.

6.2 Results

6.2.1 Identification of Affimer A2 for use in affinity purification experiments

To determine which Affimer proteins had the potential for use in an affinity purification application, pull down assays were performed. These assays compared the fibrinogen-binding ability of 7 Affimers identified through phage panning against fibrinogen (described in chapter 3). Although Affimer proteins with a C-terminal cysteine were required for use in affinity purification (to allow covalent immobilisation of the Affimer to the column resin), pull down assays were performed with Affimers not containing a C-terminal cysteine. When immobilised in an affinity column, the Affimer protein's cysteine residue participates in a covalent linkage and is not free to interact with plasma proteins, therefore the cysteine-free Affimer was the most appropriate form of the protein to use in these pull down assays. Figure 6-1A and B show that all seven Affimers pulled fibrinogen out of plasma, albeit with differing success. The presence of fibrinogen in pull down elutions was confirmed in a western blot using a polyclonal fibrinogen antibody (Figure 6-1B). Additionally, turbidimetric assays were performed with the 7 Affimers and showed that all Affimers except F5 had minimal effect on clot formation and lysis (Figure 6-1C) (as described in chapter 3). The fibrinogen-binding capacity of Affimer A2, and the normal clotting profile in the presence of the Affimer indicated that A2 was able to bind fibrinogen while having little effect on the functionality of the protein. Therefore, Affimer A2 was chosen to be immobilised in a column for affinity purification of fibrinogen.

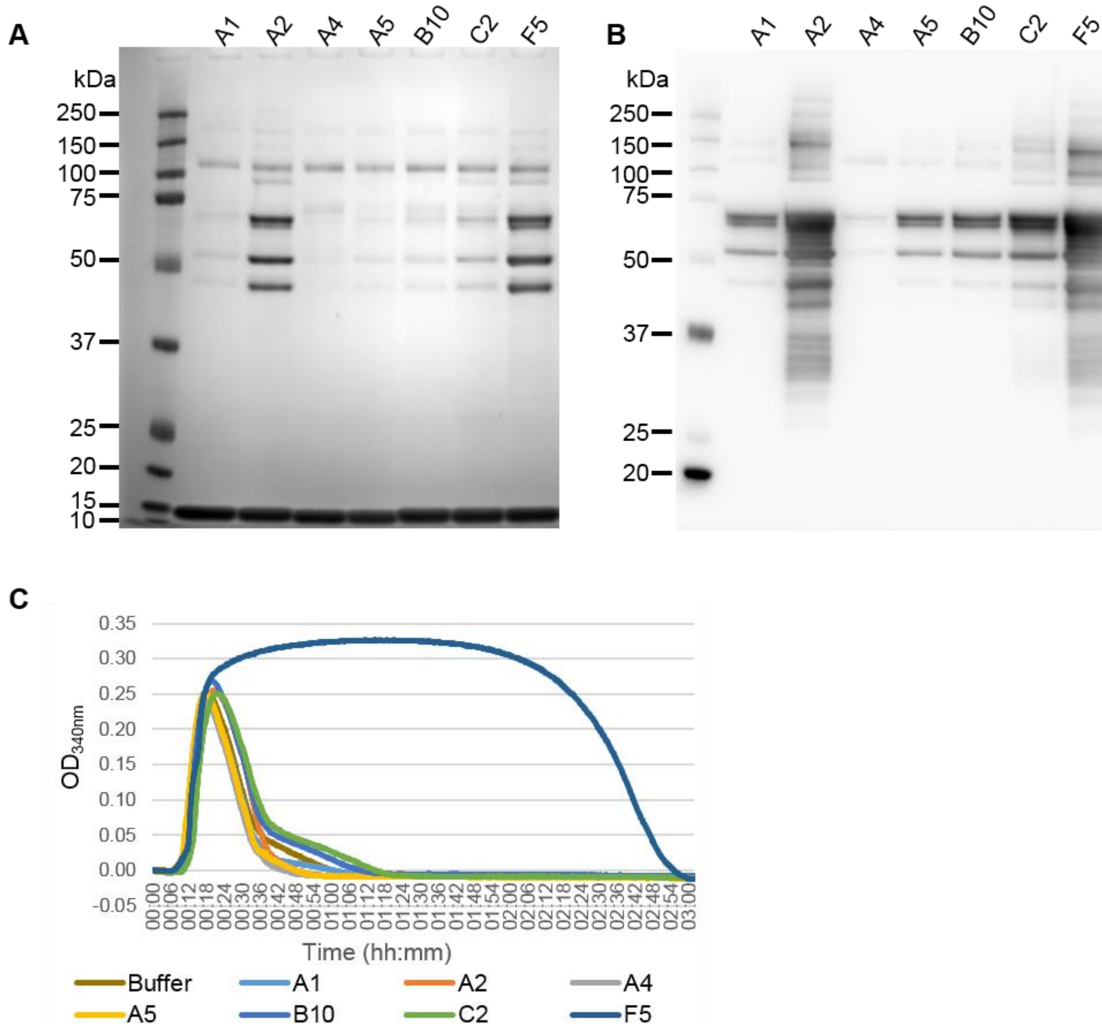


Figure 6-1 Selection of Affimer A2 for use in affinity purification experiments. A pull down was performed using Affimers (not containing a C-terminal cysteine) in plasma. **A.** Elutions containing Affimer-interacting protein were analysed by SDS-PAGE and **B.** Blotted with a polyclonal anti-fibrinogen antibody. **C.** Turbidity and lysis (T&L) assays were performed using the Affimers in plasma to determine if the Affimers had an effect on clotting parameters, indicating an effect on fibrinogen structure/function. n=3, representative trace shown.

6.2.2 Use of Affimer A2 in an affinity column for the purification of fibrinogen

The following work was performed in collaboration with Fladia Hawkins, Dr Nikoletta Pechlivani and Dr Ramsah Cheah.

A column was packed with resin covalently coupled to Affimer for use in the affinity purification of fibrinogen. Fibrinogen was purified from a commercially available preparation (Calbiochem) and from normal pool plasma.

6.2.2.1 Purification of commercially available fibrinogen

Of 2.5 mg fibrinogen loaded onto the column, a total of 0.8 mg fibrinogen was eluted. Elutions from the affinity column were analysed by SDS-PAGE, Figure 6-2A shows that fibrinogen was successfully eluted from the affinity column. A small quantity of Affimer protein was also liberated from the column during the purification protocol (circled, Figure 6-2A).

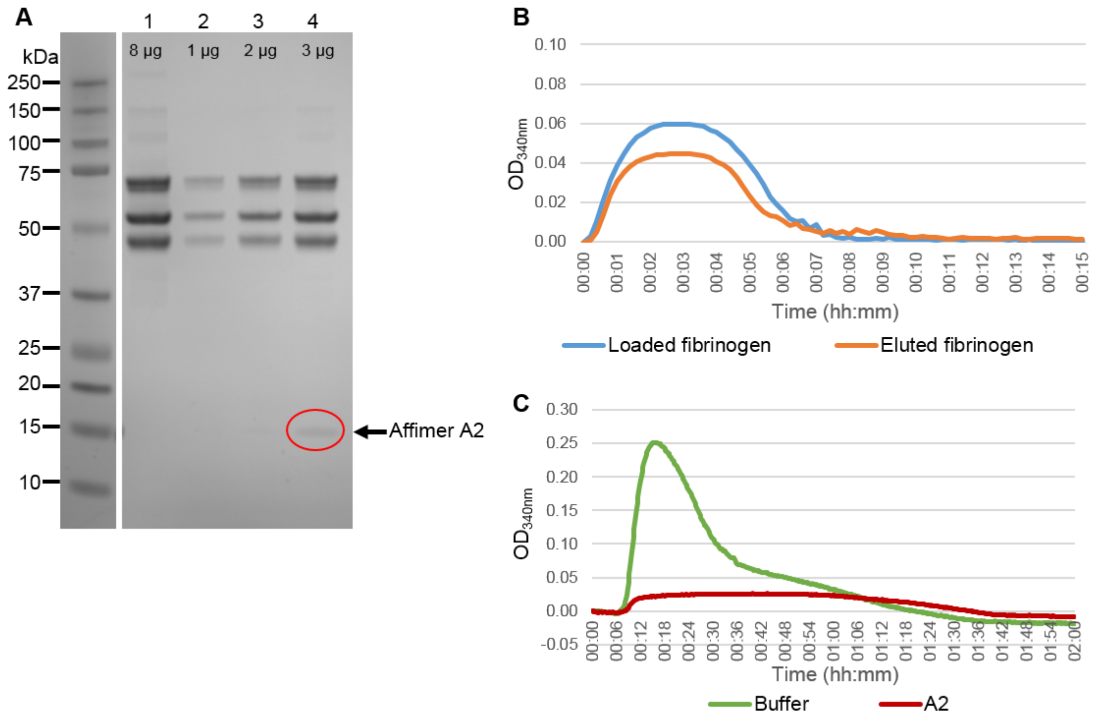


Figure 6-2 Use of Affimer A2 for affinity purification of commercially available fibrinogen. Affimer A2 was used to create an affinity column for purification of fibrinogen from a commercially available fibrinogen preparation. **A.** Following elution of fibrinogen from the affinity column, 20 µl of each elution was run on a gel. Lane: 1) Calbiochem fibrinogen (loaded), 2) Elution 1, 3) Elution 2, 4) Elution 3. Number above each lane refers to approximate quantity of fibrinogen loaded onto gel. Arrow indicates presence of Affimer protein in elution 3. **B.** Eluted protein was used in a turbidity and lysis (T&L) assay to compare the functionality of protein before (loaded) and after (eluted) affinity purification. Due to the low fibrinogen concentration in elutions, the final concentration of fibrinogen in T&L assays was 0.2 mg/ml. **C.** T&L assay in plasma demonstrating the effect of Affimer A2 (containing a C-terminal cysteine) on clotting with buffer only control. Affimer was used at 5:1 Affimer:fibrinogen molar ratio. n=3, representative traces shown.

Eluted fibrinogen was tested for functionality in a turbidity and lysis assay and compared with fibrinogen not run through the affinity column. Eluted fibrinogen clotted similarly but had a lower maximum OD (Figure 6-2B) when compared with loaded fibrinogen. The presence of Affimer A2 in protein elutions may have been responsible for the altered clotting profile of the eluted fibrinogen. As described in chapter 3, Affimer A2 (containing a C-terminal cysteine) has significant impact

on the functionality of fibrinogen, resulting in significantly reduced maximum absorbance and prolonged lag time (Figure 6-2C).

6.2.2.2 Purification of fibrinogen from plasma

An experiment similar to that described above was also performed with normal pool plasma in place of commercially available fibrinogen (Calbiochem). Normal pool plasma containing approximately 1.8 mg fibrinogen was loaded onto the affinity column, and around 1.1 mg of fibrinogen was eluted. Again, elutions from the column were examined by SDS-PAGE to determine the purity of the eluted fibrinogen. Figure 6-3A, lane 7 shows that the plasma-eluted fibrinogen was of a similar purity to commercially available Calbiochem fibrinogen (Figure 6-3A, lane 8). Eluted fibrinogen was tested for functionality in a turbidity and lysis assay, and was found to form clots with a lower maximum OD and longer lag time, indicating impaired functionality when compared with the commercially available fibrinogen preparation (Figure 6-3B).

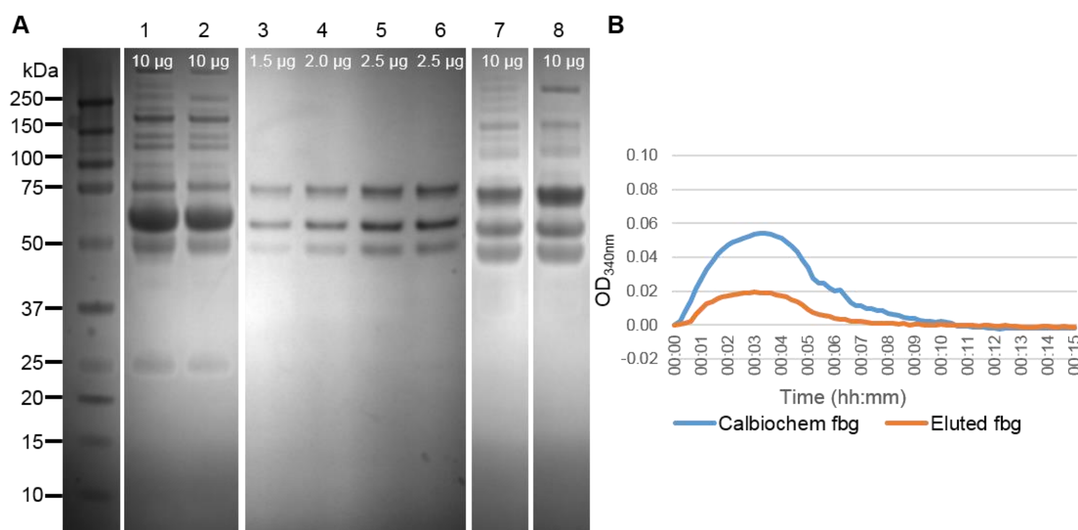


Figure 6-3 Use of Affimer A2 for affinity purification of fibrinogen from normal pool plasma. Affimer A2 was used to create an affinity column for purification of fibrinogen from plasma. **A.** Following elution of fibrinogen from the affinity column, 20 µl of each elution was run on a gel. Lane contents: 1) Plasma (loaded), 2) Non-bound plasma (flow through), 3) Elution 1, 4) Elution 2, 5) Elution 3, 6) Elution 4, 7) Pooled and concentrated elutions, 8) Calbiochem fibrinogen. Number above each lane indicated approximate quantity of fibrinogen loaded on to gel. **B.** Eluted protein was run in a turbidity and lysis assay to compare its functionality with Calbiochem fibrinogen. Due to low fibrinogen concentration, elutions were run in a T&L at a final concentration of 0.2 mg/ml.

6.2.3 Use of fluorescently labelled Affimer for visualisation of fibrin networks with confocal microscopy.

This work was designed by myself and Fraser Macrae, and confocal experiments performed by Fraser Macrae.

Affimer A2 (not containing a C-terminal cysteine) was fluorescently labelled for use in confocal microscopy. To determine if the labelled Affimer had any impact on fibrin clot structure (which would render the Affimer unsuitable for this application), clots were formed from normal pool plasma in the presence and absence of the Affimer. Figure 6-4A shows that clots formed with the Affimer had a similar structure to clots formed without the Affimer. To investigate the level of fibrinogen coverage with the fluorescently labelled Affimer, Affimer-labelled clots were compared with clots in which the fibrin had been labelled with traditionally used Alexa Fluor fibrinogen. Clots were made in the presence of both Alexa Fluor-594 fibrinogen (red) and Alexa Fluor-488 labelled Affimer (green). Figure 6-4B shows that the Affimer co-localised with traditionally used Alexa Fluor fibrinogen, confirming that the Affimer was localising to the fibrin fibres comparatively to the commercially available fluorescently labelled fibrinogen.

The labelled Affimer A2 was then used to visualise clot structure using plasma samples from individuals with hypofibrinogenemia and dysfibrinogenemia. Figure 6-4C shows that the Affimer was successful in allowing visualisation of the fibrin clot structure in these patient samples.

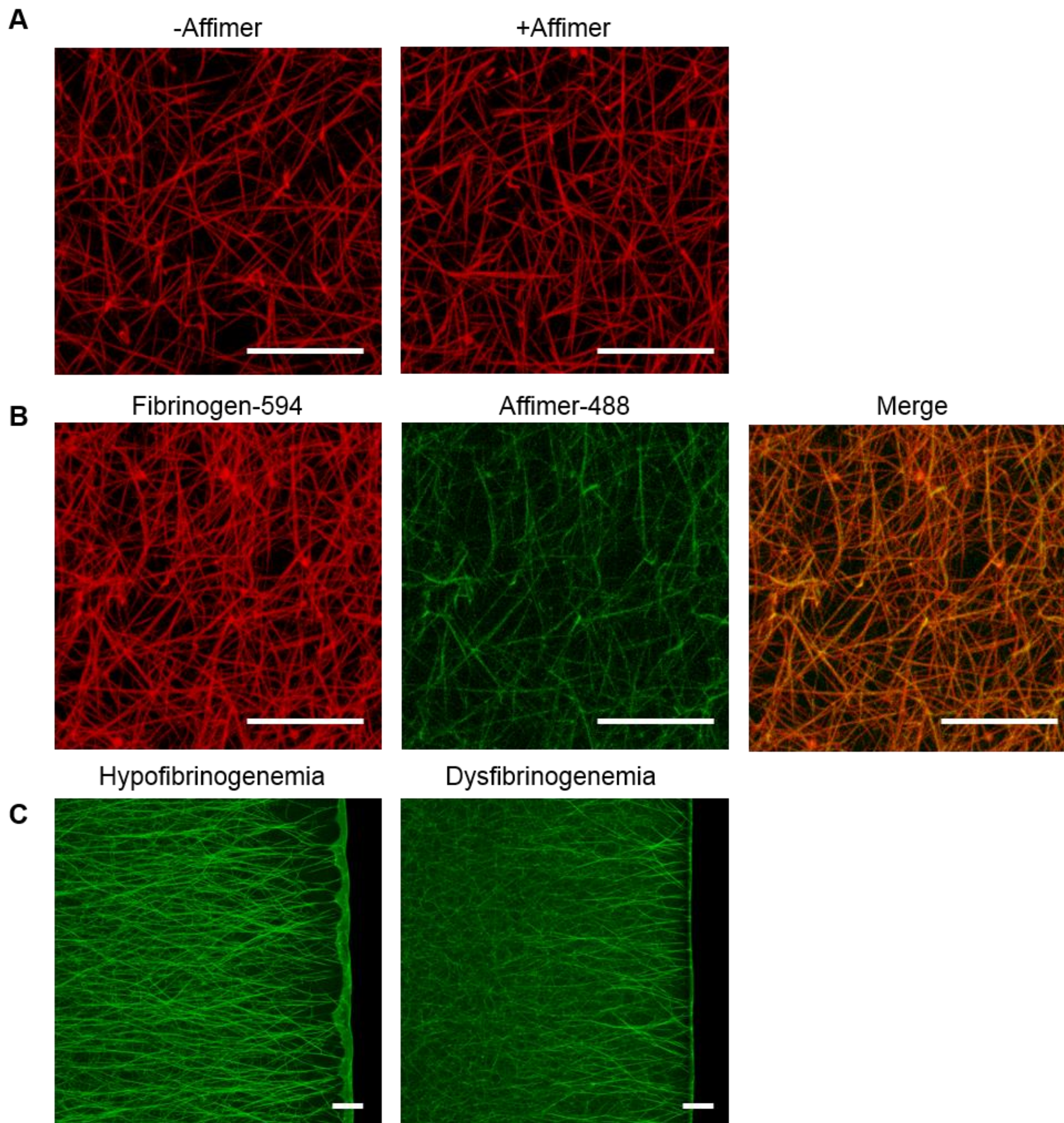


Figure 6-4 The use of Affimer A2 for visualisation of fibrin clots with confocal microscopy. A. To determine if the presence of Affimer A2 altered fibrin clot structure, clots were prepared for confocal microscopy with the addition of Alexa Fluor-594 labelled human fibrinogen (red), either in the absence (left) or presence (right) of fluorescently labelled Affimer A2. **B.** To ensure that the labelled Affimer was localising to fibrin, and providing sufficient fibrin coverage, plasma clots were prepared with the addition of Alexa Fluor-594 fibrinogen and Alexa Fluor-488 Affimer, and the co-localisation (merge) of the signals was visualised. **C.** To validate the use of labelled Affimer in the imaging of patient samples, labelled Affimer was added to clots prepared with plasma from patients with hypofibrinogenemia and dysfibrinogenemia. Z stacks of 30 images over 20.30 µm were taken, 3D images of compiled Z stacks are presented. Scale bar = 20 µm.

6.3 Discussion

6.3.1 Affimer protein for affinity purification of fibrinogen

An affinity column was produced in which Affimer A2 was immobilised to resin via covalent linkage with the Affimer protein's C-terminal cysteine. Alternative strategies for Affimer immobilisation included amine coupling or linking via carboxyl groups. However, it was possible these immobilisation techniques would target residues in the Affimer's variable regions, tethering the Affimer to the resin via the variable region loops. Use of the Affimer's C-terminal cysteine ensured that only one site on each Affimer protein was linked to the resin, leaving the remaining Affimer structure, including the variable region loops, to participate in fibrinogen binding.

The production of an affinity column was successful, and the immobilised Affimer was capable of isolating fibrinogen from both a commercial preparation of fibrinogen and from pooled plasma. Turbidimetric assays demonstrated that eluted fibrinogen had impaired functionality, with prolonged lag time and lower maximum turbidity. Compared with Calbiochem fibrinogen, SDS-PAGE showed that Affimer-purified fibrinogen was comparable in purity and so the observed effects on lag time and final clot turbidity may be explained by a small amount of contamination with Affimer A2 (harbouring a cysteine residue), which is known to alter clotting characteristics in this way (see chapter 3 for full details). Therefore, further work is required to eliminate leaching of Affimer protein from the column during affinity purification through optimisation of elution conditions. Another drawback of this method was low elution efficiency, particularly in purification of fibrinogen from Calbiochem stock, where only 32% of loaded fibrinogen was recovered. The functionality of the eluted protein and elution efficiency require improvement and there are a number of modifications to the affinity purification protocol that have been considered. The manufacturers of the SulfoLink kit recommend that neutral pH with high salt, or high pH elution conditions should follow low pH conditions if these yield protein with impaired functionality. The use of low pH during fibrinogen purification protocols is currently used by other groups in the isolation of fibrinogen γ' [377] (with no detrimental effects on fibrinogen functionality) and therefore drastic impairment of function of Affimer-purified fibrinogen is unexpected. Thus, further work is needed to determine i) whether

elution conditions can be improved to increase yield and purity of fibrinogen ii) whether contamination of eluted fibrinogen with Affimer A2 is the primary cause of the altered clotting profiles, and how this can be prevented.

The success of the widely used IF-1 monoclonal antibody method for fibrinogen purification [317, 335] lies in the fact that the antibody binds specifically to fibrinogen in the presence of calcium. Thus, with the IF-1 method, elution does not require harsh conditions, only the presence of EDTA to chelate calcium, inducing structural change in fibrinogen necessary for its release by the antibody. It may be that more detailed analysis of the A2-fibrinogen interaction would shed light on the binding, allowing optimisation of elution conditions. As mentioned previously, Affimer A2 was picked from a very limited pool of Affimers (described in chapter 3). It may be that another Affimer, identified through additional panning, would be more suited to immobilisation and use in affinity purification of fibrinogen. An ideal Affimer for this application would have a fast rate of fibrinogen binding, would stably (but reversibly) bind fibrinogen, and allow elution of bound fibrinogen without harsh buffer conditions which risk damaging protein functionality.

Affimer proteins represent a legitimate alternative to antibodies in affinity chromatography purification of fibrinogen. Affimer proteins have the ability to interact with target protein in a highly specific manner, with high affinity, and are robust enough to withstand the conditions often required during protein elution and column regeneration (e.g. extremes of pH). Additionally, Affimer proteins are easy and relatively cheap to produce in large quantities without the use of animals. There is more work to do before Affimer proteins can be routinely used for fibrinogen purification, but my data thus far represent a proof of concept supporting such an approach.

6.3.2 Affimer for imaging of fibrin clots with confocal microscopy

Affimer A2 was fluorescently labelled and its use as a fibrin(ogen) labelling reagent in confocal microscopy was investigated. Fibrin networks are typically imaged by confocal microscopy after forming clots in the presence of fluorescently labelled commercially available fibrinogen at a concentration of approximately 5% total fibrinogen concentration [247, 378]. Fibrinogen

concentration impacts fibrin clot structure, an effect which would be particularly marked in plasma containing low levels of fibrinogen, such as in individuals with hypofibrinogenemia. Additionally, in individuals with dysfibrinogenemia, the addition of wild type Alexa Fluor fibrinogen has the potential to alter the phenotype of patient fibrin networks during imaging. Regardless of whether imaging patient plasma or normal pool plasma, there is potential for the use of labelled fibrinogen-specific Affimers in the imaging of fibrin clots as it does not rely on adding additional fibrinogen during sample preparation.

Although Affimer A2 was successful at allowing fibrin clot structure in patient plasma to be visualised, there were high levels of background fluorescence during sample imaging, presumably from dye molecules not bound to the Affimer, or labelled Affimer which was not bound to fibrinogen. Although the protocol for fluorescent labelling of Affimer protein included a step for removal of unbound dye, this was clearly insufficient to remove all unbound fluorescent dye molecules. Additionally, fibrin coverage by the Affimer was comparable, but appeared to be less extensive when compared with that of commercially available Alexa Fluor fibrinogen. This is likely to be because Alexa Fluor fibrinogen is incorporated into clots uniformly, and each fibrinogen molecule is labelled with approximately 15 dye molecules. In contrast, there were only one or two fluorescent dye molecules per Affimer protein. It is likely that most Affimers will bind fibrinogen 1:1, or 1:2 (fibrinogen:Affimer), unless binding to a repeating motif in the fibrinogen structure (in which case many Affimer molecules would bind a single fibrinogen molecule), but this seems unlikely given the fibrin coverage in confocal images.

As mentioned previously, the choice of Affimer A2 for use in confocal microscopy was from a limited pool of Affimers, and it is likely that other Affimers identified through new screens would prove more useful for this purpose. Ideally, an Affimer for fibrin(ogen) imaging applications would bind to fibrinogen with high affinity, with no dissociation once bound, and have no effect on fibrin(ogen) structure. Finding an Affimer with such properties would require a new large scale screen of the phage library for fibrinogen-binders. Affimer-fibrinogen binding kinetics can be determined with SPR, and functional/structural assays would be required to

ensure that the Affimer could bind to fibrinogen without altering its structure or function.

In summary, these data show that fibrinogen-binding Affimers have potential uses as reagents in affinity purification of fibrinogen and for visualisation of fibrin clot structure in confocal microscopy. However, further optimisation is required before widespread use of Affimers for research purposes, and this is currently ongoing.

Chapter 7 Conclusions and future directions

The structure of the fibrin network and its breakdown have been the focus of much research, as clot susceptibility to lysis has implications in both bleeding and thrombotic disorders. Vascular occlusive disease, including myocardial infarction, stroke and venous thrombosis, remain the main cause of mortality in the Western population [3]. Treatment is aimed at reperfusion to regain blood flow to the affected vessels followed by prophylactic therapy to prevent re-occlusion. Potential therapies aimed at facilitating clot lysis by targeting the fibrin network have been explored [379], and this strategy may have particular benefit in subpopulations at increased risk of thrombosis due to hypofibrinolysis. Conversely, bleeding complications following traumatic vessel injury or associated with haematological disorders characterised by clot instability, represent a significant cause of morbidity and mortality. There is risk associated with both the loss of blood, and with replacement of blood/blood components/clotting factors [1, 356, 380].

Considering the potential future therapeutic benefits of targeting the fibrin network, my work concentrated on identifying fibrinogen-specific Affimers that could be used as a tool to modulate the fibrinolytic process. Affimer proteins have the potential to be used as new therapeutic agents or aid in the identification of novel therapeutic hot spots on fibrinogen that are amenable to targeting by small molecules.

Initial work was carried out with the aim of identifying proteins able to either facilitate or limit fibrinolysis. However, Affimer proteins isolated and characterised so far have all had anti-fibrinolytic function, with none demonstrating an ability to enhance clot lysis. Thus, my work focussed on the use of Affimer proteins as part of a therapeutic approach to stabilise the fibrin network with the potential for future use in conditions characterised by bleeding. This includes following surgery or trauma and in haemophilia or other coagulation factor deficiencies in which clot instability is a cause of bleeding.

Current treatment strategies to limit blood loss associated with trauma, surgery, or in certain populations with haematological disorders include the use of anti-fibrinolytics TXA and EACA, which limit plasmin generation, protecting the fibrin clot from lysis [258, 356, 381]. Additionally, fibrin sealants can be used, which

have multiple modes of action [274]. Large volume blood loss and the associated depletion of circulating clotting factors can prevent the formation of stable fibrin networks [1, 382]. Fibrin sealants not only provide a physical barrier to blood loss, sealing the edges of a wound, they also function by providing fibrinogen, thrombin, FXIII and anti-fibrinolytics to aid in the formation of a clot which is resistant to lysis to prevent re-bleeding [272, 383]. However, making the clot too resistant to lysis has the drawback of predisposing to thrombotic events and therefore a fine balance between bleeding and thrombosis risk is required to maintain healthy haemostasis.

In genetic conditions characterised by increased bleeding such as haemophilia, treatment can be even more complex owing to the factor deficiency characterising these disorders. Treatments that replace the missing/inactive coagulation factor help to restore normal haemostatic function, and although the risk of infection from replacement therapies has drastically decreased in recent years, factor replacement remains costly and is not always sufficient to prevent bleeding [380]. Moreover, development of an immune response to “external” coagulation factors can neutralise the benefit of replacement therapy, necessitating the use of bypassing agents [380]. As a result of factor deficiency in haemophilia, there is reduced thrombin generation in the propagation phase of coagulation [188, 189] which not only affects the stability of the fibrin clot through effects on clot structure, but also through delayed thrombin mediated TAFI and FXIII activation [195-197]. Relatively recently there has been a flurry of potential therapeutic strategies being developed which target the fibrin clot in individuals with haemophilia. In addition to those aiming to increase thrombin generation through inhibition of natural anticoagulants [384], there is much interest in the use of clot stabilising agents as adjunctive therapy. This approach offers the opportunity to reduce bleeding episodes between replacement therapies, making it more convenient to patients and offering both therapeutic and economic benefits [252, 256, 262, 385].

Considering the potential benefit in targeting the fibrin network, the objectives of my work were to i) identify fibrinogen-specific Affimer proteins, ii) investigate the effects of fibrinogen-specific Affimers on fibrinolysis, iii) characterise the

mechanistic pathways for Affimer-mediated modulation of clot lysis and explore the role of these proteins as therapeutic reagents.

Isolation of type II fibrinogen-binding Affimers

I was successful at isolating fibrinogen-binding Affimers from a large phage library by screening against purified fibrinogen, which were first tested for an effect on clot formation and lysis using turbidimetric assays. These assays were optimised for this work and future work with Affimer proteins.

The initial Affimer work described in chapter 3 uncovered a variety of obstacles to working with these proteins in the context of clotting. The subcloning of Affimer proteins with a C-terminal cysteine following phage panning demonstrated that this modification of the Affimer scaffold had a functional impact on clotting and lysis. Four out of the seven fibrinogen-specific Affimer proteins showed effects on clot properties in turbidimetric assays when they contained a C-terminal cysteine. Re-subcloning the Affimers without the cysteine revealed that in all the Affimers but one, it was the C-terminal modification that was altering fibrin clot properties. It is interesting that the presence of a cysteine in four of the Affimers gave rise to proteins with the ability to prolong lysis, but that this prolongation of lysis was different in the presence of each of the four Affimers. Additionally, the effect of the Affimer proteins on clot structure varied quite drastically. These observations suggest that it was the fibrinogen-binding location of each of the different Affimers in combination with the C-terminal cysteine modification that was responsible for the changes to clot properties. It is known that a free cysteine in fibrinogen Dusart is able to prolong clot lysis time due to the formation of covalent complexes between the modified fibrinogen and albumin, resulting in clots with thinner fibres, and reduced tPA-mediated plasminogen activation on the fibrin surface [213, 217, 218]. The disulphide bond between Dusart fibrinogen and albumin is also thought to be responsible for steric hindrance of plasminogen and tPA binding to their αC binding sites [84, 213]. It is possible that the fibrinogen-binding Affimers were able to form disulphide bonds with plasma proteins, and sterically hinder interaction of fibrinogen with plasminogen/tPA/other plasma proteins required for normal fibrin clot formation and lysis. Although interesting, no further work was performed to characterise these C-terminal cysteine containing Affimers in the context of clotting.

Another issue was the ability of the Affimer proteins to form aggregates, which may hinder their use as therapeutic agents, an observation that has been documented by others working with Affimers [298]. Two Affimers of interest, A2 and B10 were purified further by SEC. In these particular Affimers there was no significant functional effect of their aggregation, as the monomeric forms of the Affimer proteins functioned similarly to the aggregated forms.

In terms of further work arising from this chapter, the implications of the findings described in chapter 3 have already been implemented in the work of subsequent chapters. The propensity of the Affimers to aggregate was not an issue in further work with type II Affimers because only a single Affimer from this initial phage panning was chosen for more in-depth study (F5), and it was an Affimer which showed minimal aggregation once the C-terminal cysteine was removed.

Characterisation of type II fibrinogen-binding Affimer with anti-fibrinolytic properties

The work in chapter 4 was aimed at elucidating the mechanism of action of Affimer F5. The Affimer's interaction with fibrinogen and fibrin was characterised using binding studies and showed high affinity interactions with similar affinity of the Affimer to both fibrinogen and fibrin. The ability of the Affimer to prolong fibrinolysis was consistently seen in purified, plasma, and whole blood systems. The Affimer did not rely on changes in fibrin clot structure to induce its anti-fibrinolytic effect, as it was able to prolong lysis when added to a fully formed clot (similarly to when present during clot formation). This may have implications for its potential clinical use, in instances in which the aim is to stabilise an already-formed clot in addition to prophylactic use to reduce bleeding. Affimer F5 was found to reduce plasmin generation, and further studies using turbidimetric assays confirmed the Affimer did not prolong plasmin-induced lysis, only tPA-plasminogen mediated fibrin digestion. Moreover, F5 did not have a significant effect on uPA-induced lysis, demonstrating the importance of F5 interaction with the fibrin network to exert its anti-fibrinolytic effect. Due to the finding that the Affimer bound to fibrinogen D fragment, and molecular modelling predictions that placed the Affimer close to tPA and plasminogen binding sites, it was hypothesised that Affimer F5 was blocking tPA and/or plasminogen binding to their D region binding sites. However, extensive ELISA and SPR based binding

assays have not been able to prove that F5 interferes with either tPA or plasminogen binding to fibrin DD fragment. It may be that F5 prevents tPA and plasminogen interacting on the surface of fibrin, in which case there would be no observable reduction in tPA or plasminogen binding to fibrin(ogen), only reduced plasmin generation, which was demonstrated (F5 mechanism of action summarised in Figure 7-1). Affimers have been shown by others to function via mechanisms that do not require direct binding to the active site of a target protein [386], and this may be what is happening with Affimer F5.

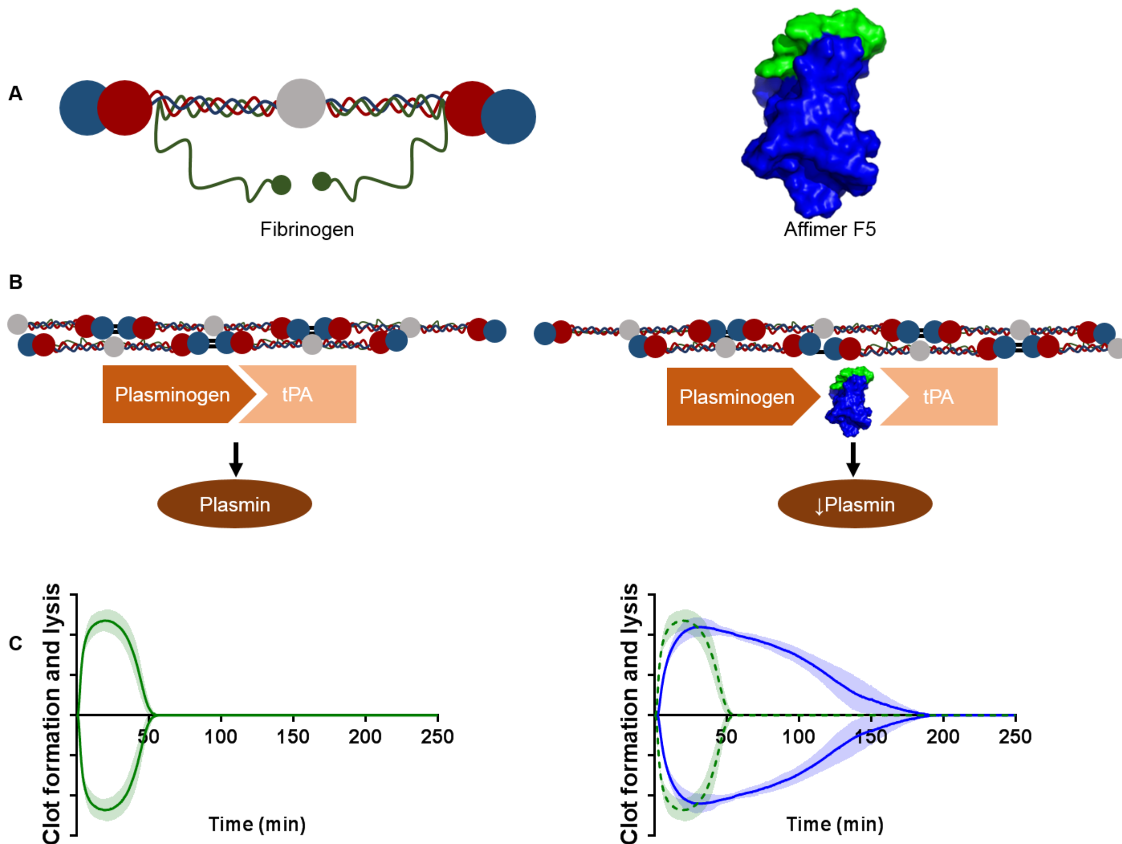


Figure 7-1 Summary of F5 mechanism of action. A. Schematic representation of a fibrinogen molecule and Affimer F5. **B.** Plasminogen and tPA assemble on the fibrin surface to produce plasmin. When fibrin(ogen) binding Affimer F5 is present, plasmin generation is reduced, due to interference with plasminogen-tPA interaction on the fibrin surface. **C.** Normal clot lysis (green trace) is therefore prolonged (blue trace) in the presence of Affimer F5, due to reduced plasmin generation.

If the mechanism of this Affimer protein is to be conclusively determined, further work is needed, and would involve crystallisation studies of the Affimer in complex with fibrin(ogen). Of course, it would be useful to co-crystallise the Affimer-fibrin(ogen) complex with tPA and plasminogen, but this poses experimental

difficulties, as these proteins cannot be united without fibrin digestion and therefore any future work will require complex studies using fibrin fragments.

As is the case with any potential therapeutic agent, further work will have to be carried out, including *in vivo* work in appropriate animal models to demonstrate the potential efficacy and safety of the Affimer protein .This is complicated by the inability of Affimer F5 to prolong lysis in mouse plasma, with the Affimer causing a slight enhancement of clot lysis. These mouse data suggest that the Affimer interacts with a different region of mouse fibrinogen than it does human fibrinogen. Animal studies using fibrinogen deficient mice supplemented with human fibrinogen would enable Affimer testing *in vivo*. First, *ex vivo* experiments should be performed with Affimer protein in plasma from fibrinogen deficient mice supplemented with human fibrinogen, to determine if such a mouse model would be appropriate to use. An additional step before proceeding with *in vivo* work could include the use of the Affimer in a flow model, to investigate whether F5 is able to function under more physiological conditions of a clot formed and lysed under shear stress. Such flow models have been described and used by others [387]. These experiments should also compare the efficacy of Affimer F5 with existing therapeutic agents, such as TXA.

As well as the efficacy of Affimer protein F5 in animal models, half-life of the protein also needs to be explored. Affimers, being only ~13 kDa, have a short half-life due to renal clearance [297, 388]. For Affimers with low affinity for their target protein, this would be problematic as the Affimer protein may be cleared from the circulation before binding to target protein (fibrinogen). There are means to prolong Affimer protein half-life, some of which are under investigation by Avacta, including fusion of Affimer proteins to albumin molecules [308]. However, it is unlikely that such a means of prolonging half-life would be suitable for Affimer F5, given its intended function. An Affimer protein conjugated to albumin (66 kDa) would have hindered movement in blood, reduced diffusion into the fibrin clot, and may experience reduced ability to bind to fibrin(ogen) at the target site. Therefore, future studies will need to fully characterise the Affimer protein's efficacy and pharmacokinetics, including half-life extension studies, if necessary.

It is also possible that the region of fibrinogen which binds Affimer F5 is amenable to targeting by small molecules, as an alternative strategy to using the Affimer protein directly. Work with Affimer F5 (chapter 4) and B3 (chapter 5) has shown that the function of Affimers cannot simply be replicated with linear peptides of the same sequence as the Affimer variable regions. Linear peptides failed to maintain the shape of the Affimer variable region loops and thus showed no effect when tested in turbidimetric assays. Identification of small molecules that are able to mimic the function of Affimer proteins will therefore be required. Molecular modelling of the Affimer protein variable region loops and virtual screening of large, commercially available compound libraries will be necessary. Small molecules which replicate the shape and electrostatic profile of Affimer variable regions can be identified with shape similarity matching software [389]. This procedure would be useful to implement in the future for Affimers of interest, to explore the possibility of small molecule effectors as an alternative to the use of whole Affimer proteins.

Modulation of fibrinolysis with fibrinogen-binding type I Affimers

Similarly to previously discussed chapters, the work in chapter 5 was performed with the aim of isolating fibrinogen-binding Affimers for modulation of clot lysis, except that the Affimers used in screening had a type I Affimer scaffold. Type I Affimers have a scaffold based on human Stefin A protein [306], in contrast to that of the type II Affimer scaffold which is based on a phytocystatin consensus sequence [289]. The rationale behind using this alternative scaffold for screening was the potential for reduced immunogenicity were any candidates to make it to human trials.

Screening type I Affimers for fibrinogen-binding yielded six Affimers in total, of which two, named B3 and B9, had an effect on clot lysis, both causing a prolongation in lysis time. Similarly to that described in chapter 3, the issue of Affimer protein aggregation arose, this time, Affimer B9 aggregated into multiple species which also associated with bacterial proteins. Unlike Affimers A2 and B10 described previously, Affimer B9 did not function in its monomeric form and the multimeric form could not be purified from the bacterial contaminant proteins, leaving Affimer B3 for further investigation.

Turbidimetric experiments determined that the prolongation of lysis by Affimer B3 was dependent on the protein's ability to change fibrin clot structure, as the Affimer only prolonged lysis of clots when it was present during clot formation and induced structural changes. Protein pull downs identified the N-terminus of the fibrinogen β chain as a potential site of B3 interaction, near to the cleavage site of FpB. The Affimer had a higher affinity for fibrinogen than for fibrin, and was not incorporated into fibrin clots as viewed by confocal microscopy, supporting the idea that the Affimer was interacting with a region of fibrinogen that undergoes conformational change upon conversion of fibrinogen to fibrin. The mode of action of Affimer B3 is summarised in Figure 7-2.

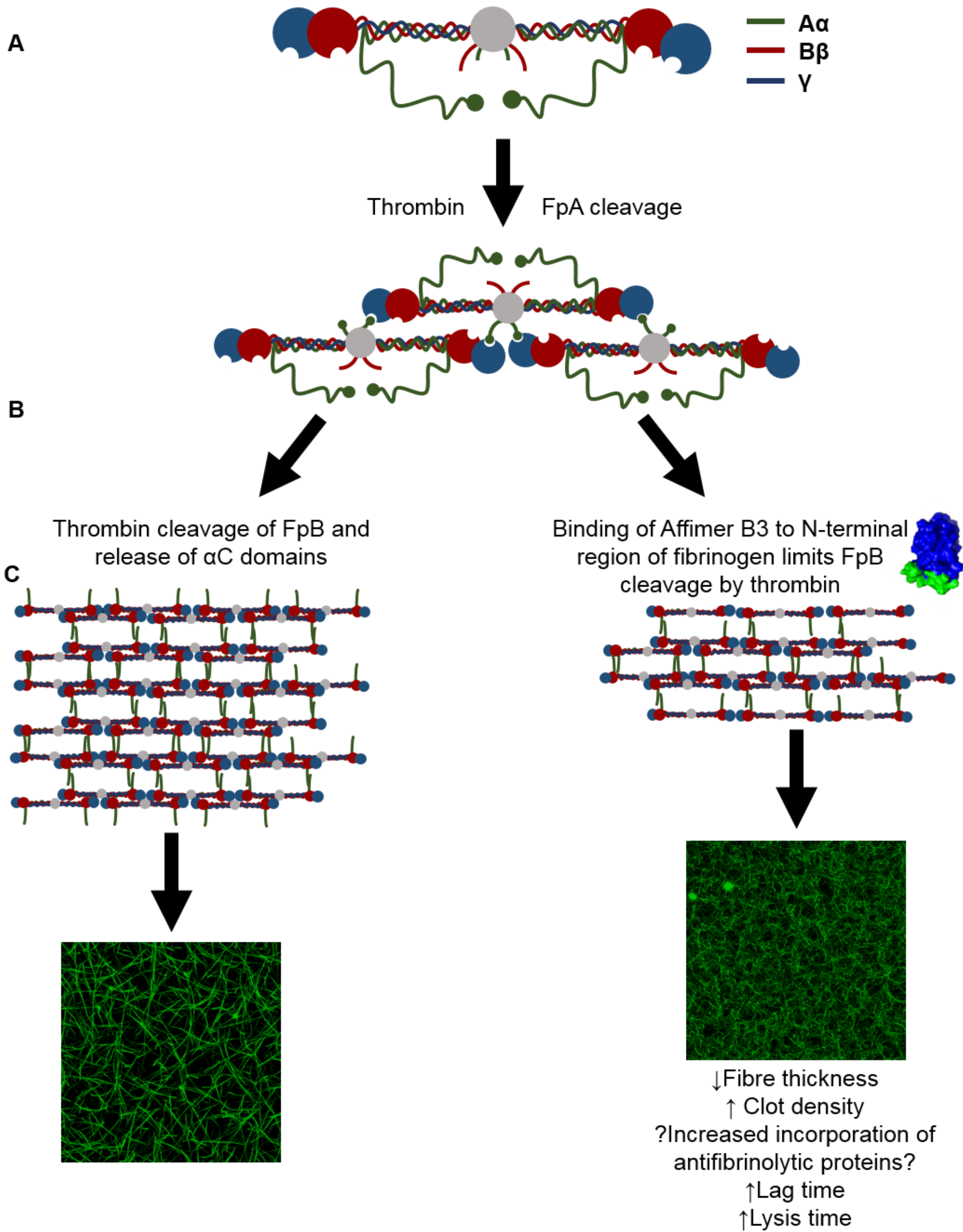


Figure 7-2 Summary of Affimer B3 mechanism of action. A. During clotting, thrombin cleaves fibrinopeptide A (FpA) from fibrinogen, allowing fibrin monomers to align and form protofibrils. **B.** Thrombin then cleaves fibrinopeptide B (FpB), which causes the release of the αC domains of fibrinogen, and intermolecular interactions between protofibrils, to form fibrin fibres. Binding of Affimer B3 to the N-terminal region of the fibrinogen β chain was hypothesised to reduce thrombin cleavage of FpB. **C.** Clot formation in the presence of Affimer B3 is altered, lag time is increased, clots formed have thinner fibres and are more dense, and lysis time is prolonged in the presence of the Affimer protein. Prolongation of clot lysis in the presence of B3 was hypothesised to be due to altered incorporation of antifibrinolytic proteins into the more dense clot formed with the Affimer.

Although interesting mechanistically, this Affimer was tested in whole blood and was found to cause a paradoxical enhancement of clot lysis, presumably due to the formation of thinner (and therefore weaker) fibres which were less mechanically stable. Alternatively, the Affimer may modulate fibrinogen-platelet interactions or even platelet activation, and further work would be required to confirm these theories. The findings described in this chapter will influence future work, in reinforcing the notion that in seeking agents which modulate fibrin clot lysis, one must ensure that the structure of the fibrin network is not significantly altered. Clots with thinner fibres (and that are also more dense) are indeed associated with hypofibrinolytic phenotype, but ROTEM data suggest that the degree to which Affimer B3 interfered with clot structure detrimentally affected the strength of the clot. Although disappointing that B3 showed no prolongation of lysis in whole blood, this ROTEM finding also demonstrated that future screens to identify Affimers for in-depth investigation should include whole blood studies early on in Affimer protein characterisation. Turbidimetric assays in plasma and purified systems are useful in high throughput evaluation of Affimers following phage panning, but if Affimers are to be characterised for the purpose of providing therapeutically relevant insight into matters of fibrin clot stability, they must show function in whole blood systems.

In addition to performing experiments with Affimer proteins in whole blood soon after their isolation, there are other experiments that could be added to the panel of assays used to characterise Affimer proteins that would shed light on their mechanisms of action. For example, since FXIII and PI have major impact on a clot's resistance to lysis [198], assays should be performed to determine if the Affimers have an effect on FXIII crosslinking of fibrin [353], or incorporation of PI into the clot [120].

Use of Affimers for protein purification and visualisation of fibrin networks

Chapter 6 described the use of Affimer proteins in alternative roles as protein reagents. Protein pull down assays performed and described in chapters 4 and 5 demonstrated the ability of Affimer proteins to be immobilised and pull fibrinogen from plasma, representing a complex mix of proteins, with relatively little contamination from other plasma proteins. In addition to this observation, it was also recognised that there is a need for a new means of obtaining highly pure

fibrinogen, as the commonly used IF-1 monoclonal antibody produced by Kamiya Biomedical [317] is no longer produced. Affimer proteins are ideal candidates for use in affinity purification because of the ability to isolate Affimers that are highly specific to the target protein, in addition to their thermal and chemical stability allowing for maintenance of function even following extended periods and stringent wash conditions in an affinity column [289, 297]. Affimer proteins can also be produced relatively easily in large quantities. Affimer A2, isolated from the initial screen of type II Affimers against fibrinogen (described in chapter 3) was investigated for this purpose. The Affimer protein was immobilised via a C-terminal cysteine and was able to purify fibrinogen from a commercially available fibrinogen preparation and also from plasma. Fibrinogen yield and protein functionality was less than optimal, and more work is required to improve the method. Future work should include characterisation of the Affimer A2-fibrinogen interaction to understand how the Affimer protein binds to fibrinogen, and therefore provide insight into the best conditions to optimise this binding, and also to reverse this interaction to allow efficient fibrinogen elution during affinity purification protocols.

The same Affimer, Affimer protein A2 was labelled fluorescently for use in confocal microscopy for the imaging of fibrin clots made from plasma from dysfibrinogenemic and hypofibrinogenemic patients. This use of Affimers in fibrin imaging of patient clots could be particularly useful as it does not rely on the addition of fluorescently labelled wild type fibrinogen for visualisation. Addition of the labelled Affimer to plasma samples prior to clot formation allowed the fibrin structures in the patient samples to be visualised, and the Affimer had a comparable fibrin coverage to commercially used Alexa Fluor fibrinogen. There were some problems with the use of the Affimer in this application, such as high levels of background fluorescence, however, optimisation of Affimer protein labelling and removal of non-bound dye could improve this.

It may be that more suitable Affimers for these sorts of applications could be isolated through additional screens, but these data provide a proof of concept for use of the Affimers in affinity purification and imaging of fibrin(ogen).

Summary of future work

Although Affimers and other engineered protein scaffolds are relatively new when compared with antibodies, they have already proven useful as molecular biology reagents in a variety of settings, for imaging, in affinity purification of proteins, and for investigating protein-protein interactions [287, 297]. Furthermore, there exist engineered protein scaffold-based therapeutics in clinical trials in humans, and proof of concept for scaffold based therapeutics has already been demonstrated by the approval of Ecallantide (Kalbitor) in 2009. Kalbitor was approved for use in hereditary angioedema and is an inhibitor of plasma kallikrein with a structure based on the Kunitz domain of human TFPI [302, 390]. There is therefore the possibility that Affimer proteins may eventually be suitable for clinical use after extensive *in vivo* work.

My work has shown that Affimers represent a tool for modulation of clot lysis. The way forward seems clear: first, further characterisation of the mechanistic pathways for F5-induced prolongation of clot lysis is required. This is likely to involve complex crystallisation studies that will provide valuable information, not only on F5-fibrinogen interaction, but they also have the potential to identify new therapeutic targets amenable to small molecule intervention. Second, in-depth studies of F5 action *in vivo* are needed using suitable animal models of both bleeding and thrombosis. This should also include pharmacokinetic, toxicology and immune studies to ensure safety of Affimers, and comparisons should be made with existing therapeutics to compare the efficacy of Affimer proteins with drugs in current use. Work may also need to be done to identify methods for prolonging Affimer protein half-life. Third, a large scale screening of latest generation Affimer library is required with binders isolated at different panning steps to create a panel of fibrinogen-specific Affimers with different binding affinities that can then be tested for their potential clinical development. It may be beneficial to also screen for fibrin-specific Affimer proteins, which have the potential to be more targeted to sites of bleeding in the context of acute injury in which a clot has already formed.

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