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Highlights

The Evolution of Fibrin-Specific Targeting Strategies

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Fibrin-specific targeting capabilities have been highly sought for over 50 years due to their implications for bio-molecule delivery, diagnostics, and regenerative medicine. Yet only recently has our full knowledge of fibrin's complex polymerization dynamics and biological interactions begun to be fully exploited in pursuit of this goal. This highlight will discuss the range of rapidly changing strategies for specifically targeting fibrin over the precursor fibrinogen and the advantages and disadvantages of these approaches for various applications.

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Introduction – Why Target Fibrin?

Fibrin is the native provisional protein matrix that forms in response to injury and plays an important role in wound healing. Recently, it has become a major focus for molecular targeting strategies because of its prominent role in a variety of pathologies including cancer, where its deposition is coupled with tumor metastasis[1] and angiogenesis[2], inflammatory conditions such as rheumatoid arthritis[3], sepsis[4], and glomerulonephritis[5], and central nervous system disorders associated with a break-down of the blood brain barrier such as Alzheimer's disease[6] and multiple sclerosis[7]. Most notably, it is a major contributor to cardiovascular disease[8] (CVD) where it is deposited in atherosclerotic plaques that can rupture and lead to stroke and myocardial infarction; such events underlie the leading cause of morbidity and mortality in Western societies[9].

Importantly, fibrin-targeting strategies have largely been centered on CVD due in part to current deficiencies in existing diagnostic and therapeutic delivery technologies. For instance, there is a major push for the targeted delivery of thrombolytics which could both minimize potentially serious hemorrhaging side effects while enhancing potency[10-12]. Further, the majority of diagnostic strategies, such as Doppler ultrasound, angiography, and contrast-MRI, measure vessel narrowing or occlusion either directly via morphological measurements or indirectly via blood flow; they fail to account for the fact that flow obstruction alone is an inadequate predictor of acute cardiovascular events like stroke and pulmonary emboli[13, 14]. In particular, vulnerable plaques located in vessels with only 50 to 60% residual stenosis constitute the most common source of thromboembolism[15].

Molecular targeting of fibrin is not a novel concept, with some earlier attempts dating to the 1960's, although the robustness and success of many strategies has been hampered by the

challenge of distinguishing fibrin from its precursor fibrinogen, a protein present at concentrations of 2-4mg/mL in the blood[16] and which shares 98% of its structure with fibrin[17, 18]. Therefore, this Highlights article will focus exclusively on fibrin-targeting strategies, summarized in Table 2, that pointedly differentiate the two proteins. While the physical structure of fibrin-targeting entities is important and will be briefly discussed, the focus of this highlight is on the various functional fibrin-targeting strategies and how these have been exploited for various applications.

Molecular Structures of Fibrin-Targeting Entities – Antibodies, Antibody Fragments, and Small Peptides

Among the earliest and most straightforward molecular structures chosen to target fibrin were those of full-length antibodies. Antibodies constitute the immune system's intrinsic molecular method for achieving a diverse multitude of pathogen-derived antigen binding specificity. They consist of two regions—variable (Fv) and constant (Fc)—that are made up of heavy peptide chains that extend the entire length of the molecule and light chains that are much shorter (Figure 1). The functions of the variable and constant regions, respectively, are to effect antigen-binding and host-cell recognition. The main advantage of antibodies is their capacity for high ligand affinity – often within the low nanomolar range—conferred in part by their intrinsic bi-valent nature and *relative* stability owing to the four disulfide bonds through the length of the molecule[19].

Antibodies also possess several deficits. They tend to be large, often exceeding 100 kDa, therefore resulting in long circulation times, which for imaging applications may result in excessive amounts of background signal and impractically long imaging windows. Antibodies are usually derived in mice or other non-human species, and because the constant region may be recognized as a T-cell-stimulating epitope, such approaches elicit immunogenicity concerns in humans[20, 21]. Further, they can possess low solubility, a tendency to aggregate, and sensitivity to temperature and pH, all of which are detrimental to long-term stability[15, 17]. Due to these deficiencies, several promising fibrin-specific antibodies produced as early as the 1980's[17, 22, 23] have been modified to antibody fragments and are still in wide-spread use as targeting strategies[10, 14, 24-26].

Antibody fragments (AFs) are smaller portions of antibodies that retain specific ligand-binding capabilities but with lower immunogenicity concerns[27]. Their smaller molecular weights compared to full length antibodies also confers shorter circulation times, the ability to penetrate deeper into fibrin structures, and the facilitation of their

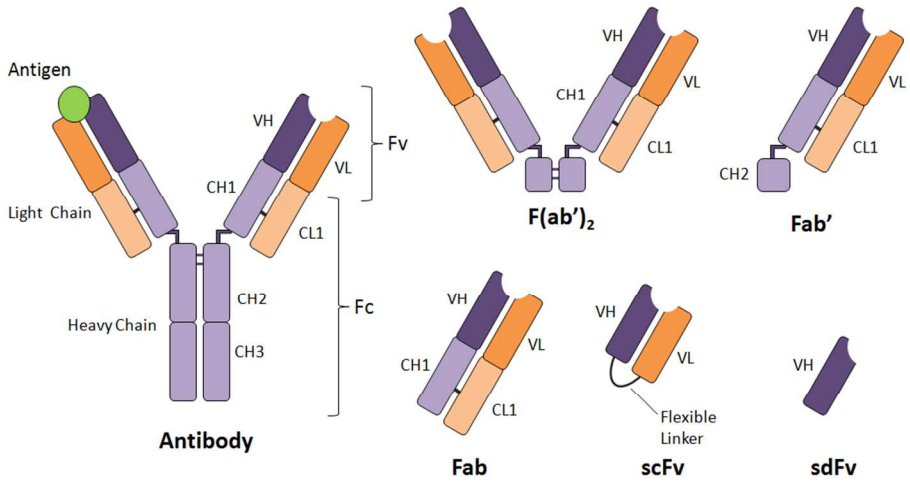

Figure 1: A cartoon illustration of an IgG antibody and the nomenclature for some of its various possible fragmented compositions. Heavy and light chain components are depicted in purple and orange, respectively.

production in prokaryotic expression systems, the latter of which can lower production costs[28].

AFs come in a range of sizes, the largest being $F(ab')_2$ and then Fab' fragments, which, respectively, are divalent and reduced monovalent versions containing complete antigen binding regions plus a small portion of the constant region below the "hinge" of the antibody[27]. Importantly, a study by Tjandra *et. al*. demonstrated the ability of murine-derived $F(ab')_2$ fragments to generate human anti-mouse antibody responses (HAMA) in some rare instances after only a single dose, and very commonly after repeated doses[21]. This indicates that the small portion of the constant region present in both $F(ab')_2$ and Fab' fragments still represents a legitimate immunity concern.

Smaller yet are Fab fragments, single-chain variable fragments (scFvs), and single-domain variable fragments (sdFvs), all of which lack the heavy chain Fc region that lends to further elimination of immunogenicity concerns. ScFvs are the smallest AFs retaining complete antigen-binding sites. Though their two components may artificially be linked together via crosslinking or engineered peptide linkers, their lack of natural di-sulfide bonds potentially lowers both their stability and ligand-specific affinity. SdFvs are the smallest AFs with any amount of ligand-specific affinity, though this tends to be lower than that of larger fragments with intact binding sites. Importantly, the abundance of fibrin as a target, present at 20-200mM in thrombi[29], may render even low-affinity entities effective targeting agents. Furthermore, compromised binding affinities may be enhanced by constructing bi-valent AF species or attaching multiple copies of an AF to a delivery vehicle.

Peptides tend to be even smaller than AFs, and they thus possess commensurately greater advantages in all the aforementioned characteristics associated with small size. They possess the lowest production costs and typically minimal to no immunogenicity concerns[30]. Their main disadvantage is their possession of binding affinities below those of AFs – typically in the micromolar range—though these can likewise be improved through an increase in valency, such as epitomized by Obermeyer et. al.[31] who conjugated 90 copies of the tripeptide GPR to the outside of a viral delivery capsid. Another potential disadvantage of small peptides is that while they may possess binding specificity for fibrin over fibrinogen, the smaller the sequence, the more likely it is to also possess binding capabilities for additional biological molecules; for instance, GPR, mentioned above, is also a ligand for αxβ2 integrins[32].

Functional Fibrin-Targeting Strategies

The general strategies for distinguishing fibrin from its fibrinogen precursor can be divided into the following rough categories: the "A Priori Approach" whereby previously-discovered specific distinguishing fibrin epitopes are used as ligands, the "Shotgun Approach" whereby cross-linked fibrin clots are fragmented and used as ligands, the "Biological Mimicry Approach" whereby epitopes of naturally-existing molecules known to bind fibrin are transformed into recombinant analogs, and the "Evolutionary Approach" whereby phage libraries are used for high-throughput panning against whole fibrin structures or fibrin epitopes.

The main exception to these general categories is radioactively labeled fibrinogen, the fairly straightforward concept introduced in the 1970's whereby full-length 340 kDa fibrinogen molecules labeled with I^{125} were expected to become incorporated into forming thrombi[33]. It was commonly used into the 1990's for its promising sensitivity in detecting deep vein thrombosis (DVT) in the calf region[24]. However, long circulation times and overall low specificity for all venous thromboses combined have since rendered it obsolete[34]. A summary of fibrin-targeting efficacy for radio-labeled fibrinogen and other entities tested in humans is provided in Table 1.

The A Priori Approach

Understanding the logic of the "A Priori Approach" requires a brief discussion of fibrinogen conversion into cross-linked fibrin. The conversion process begins when the serine protease thrombin cleaves the N-termini of fibrinogen's symmetrical Aα and Bβ chains, both located within the central N-terminal disulfide knot, or NDSK region, a.k.a. 'E Region', producing new residues referred to as knobs 'A' and 'B', respectively, that bind to complementary polymerization 'holes' in the distally located D Regions to produce protofibrils. The two knobs 'A' are exposed more rapidly than knobs 'B', and thus they are thought to play a more prominent role in protofibril formation, whereas knobs 'B' are thought to be more important for the lateral aggregation of protofibrils[16, 18]. Activated transglutaminase factor XIII (FXIIIa) subsequently crosslinks adjacent D Regions within a protofibril forming DD-Dimers. When fibrin is ultimately digested by plasmin, the resultant products are DD(E) complexes, E-Fragments, and DD-Dimers. Through the "A Priori Approach" fibrin-targeting entities have been developed to target knobs 'A'[35-37], knobs 'B'[17, 22, 38], E-Fragments[39], and DD-Dimers[23].

These molecular targets each possess distinct advantages and drawbacks. While crosslinked DD-Dimers can constitute fibrin specific structures, once a clot is degraded, they are likely to disperse throughout the vasculature as free DD-Dimers. DD-Dimer presence in blood serum is, in fact, a common clinical assessment of deep vein thrombosis (DVT), and the extensive data collected on its utility has brought to light a disturbingly high rate of false positives with specificity tending to range between 40 to 60%. Conditions associated with significantly high rates of serum DD-Dimers include rheumatoid arthritis, sickle cell disease, asthma, whole-body or limb-immobility, recent surgical exposure, pregnancy, and old age. Ninety six percent of pregnant women in their third trimester tested

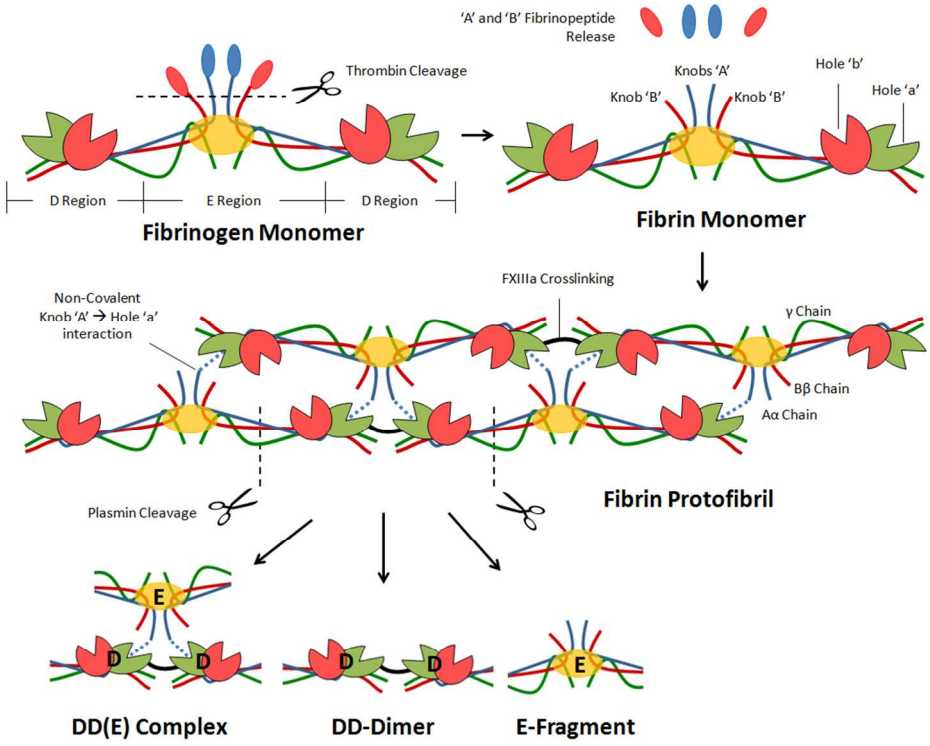

Figure 2: A schematic representing the conversion of fibrinogen to fibrin, subsequent assembly of fibrin monomers into a protofibril, and finally, plasmin-mediated fibrin degradation and resultant products. Alpha, beta, and gamma chains are depicted in blue, red, and green, respectively.

positive for DD-Dimers, and the percent of elderly patients testing positive for DD-Dimers increases from 58% for those 60-69 to 83% for anyone above 80[40] .

The concern with choosing either DD-Dimers or E-Fragments as a target is therefore the potential for non-specific uptake of the targeting entity, which may be especially problematic for bio-molecule delivery applications. Non-specific uptake may be overcome for imaging applications by simply accumulating sufficient signal at pathological sites to distinguish from background, although baseline DD-Dimer levels are likely to vary among patients therefore making determination of a standardized imaging window more difficult. Despite these challenges a DD-Dimer-targeting mAb isolated by Rylatt et. al.[23]and subsequently converted to both a $F(ab')_2$[41] and a Fab' format called ThromboView® has shown great promise in phase II clinical trials for detecting DVT[42] (Table 1). The main drawback was significant levels of background signal that required taking additional baseline images three hours apart from diagnostic images.

Knobs 'A' and knobs 'B' also constitute fibrin-unique targets, although a key difference from DD-Dimers is their intrinsic importance to the active fibrin polymerization process that makes them most prominently available during clot formation. Entities targeting either of the knobs are therefore likely to exhibit a strong targeting preference for newer clots or those only in the active process of polymerization[43]. Depending on the application, this fact may either limit efficacy or provide a useful tool for distinguishing different categories of fibrin targets. More troubling, however, is the implication that anticoagulant therapies such as heparin, which are designed to impede clot polymerization, may therefore also interfere with the targeting ability of entities specific for fibrin knobs[38]. This concern is difficult to ignore, especially considering that

Table 1

Entity	Molecular Target	Pathological Target	Sensitivity	Specificity	Sample Size	Source
I^{125} Fibrinogen	Fibrinogen	All venous Thrombi	45%	92%	2484	[34]
		Distal DVT	Up to 92%	NA	88	[45]
Thomboview ® (^{99m}Tc-labled 'Fab)	DD-Dimer	Proximal DVT	84.2%	97.6 %	66 (phase II clinical)	[42]
EP-2104R (Gd-labeled peptide)	DD(E) Complex	Venous Clots	67%	NA	6 (phase II clinical)	[46]
		Arterial Clots	80%	NA		
Fibronectin N-terminal FBD (^{99m}Tc-labeled peptide)	Fibronectin -Binding Domain of Fibrin	Lower Limb DVT	80-93%	NA	40 (phase II clinical)	[47]
C22A (^{111}In-labled 59D8 Fab)	Knob 'B'	Thrombotic Disease	85%	100% (n= 10)	34	[26]
		Distal DVT	86%	100% (n=10)	28	
^{99m}Tc-labelled T2G1 Fab'	Knob 'B'	Arterial Thrombi	50%	NA	18	[44]

Table 1: An overview of the most up-to-date efficacy results for each of the fibrin-binding entities tested in humans. NA = Not available. Sample sizes used for sensitivity and specificity calculations correspond to the "sample size" column unless otherwise noted.

many patients possessing, or suspected of possessing, some sort of fibrin-associated pathology are likely to be prescribed an anti-coagulant regimen.

Nevertheless, knob 'B' targeting strategies have proven useful in some CVD applications. Of the most widely-used knob 'B' targeting entities are 59D8 and T2G1, originally isolated as mAbs by Hui et. al,.[17] and Kudryk et. al.[22], respectively. They have subsequently been modified to Fab', Fab, and scFv formats, and their binding affinity for knob 'B' has been optimized via phage display[10]. Variations of 59D8 and T2G1 have shown promise as a targeted anticoagulant therapy[10], and highly-accurate CVD diagnostics that have been tested in humans[24-26, 44] (Table 1), though notably, concerns have been raised regarding their performance in the presence of anticoagulants[14].

The Shotgun Approach

By design, the "Shotgun Approach" is less conscientious in its implementation by comparison to the "A Priori Approach." The method essentially works by immunizing mice to freeze-fractured fibrin clots and collecting the resultant antibodies. The main detriment to this approach is that there's no guarantee the resultant antibodies will actually bind to a fibrin-unique epitope, since again, fibrin and fibrinogen are 98% identical, and so screening for fibrin-specificity will certainly be required at some point. Once an antibody that possesses high affinity and specificity for fibrin is isolated, several additional assays would be required to determine where on fibrin the antibody actually binds, if the specific site is determined to be important.

Very often, the resulting antibody shares any of a number of epitopes previously identified in the "A Priori Approach". This was the case with NIB 5F3 which was eventually determined to specifically bind the E-Fragment of fibrin[48], and the MH-1 antibody[49] that was eventually determined to bind DD-Dimer[50]. Additionally, it is possible to identify an antibody through this method that specifically targets fibrin but is also capable of targeting a fibrin degradation product. This is the case with NIH 1H10[48] which possesses an affinity for fibrin of K_D = 3.9nM, though it also possess a strong affinity for the E-Fragment (K_D = 8.04nM). Despite this drawback NIH 1H10 has been successfully used to detect fibrin clots as small as 500μm in diameter in canines using MRI[15], as well as to enhance clot dissolution in a canine femoral artery injury model[51].

Certainly, though, the "Shotgun Approach" does produce some truly fibrin-unique binders that would not have been possible utilizing the "A Priori Approach" alone, as demonstrated by *Hisada et. al.* with their mAb that targets residues 149-234 of the fibrin Bβ chain, and that does not bind to any fibrin or fibrinogen degradation products [50]. In its debut study, this mAb showed promise for diagnostic imaging of gliomas in rats.

The Biological Mimicry Approach

The Mimicry Approach seeks to emulate the fibrin-binding capabilities of various existing peptides and enzymes by building recombinant protein imitations of the amino acid sequences important for fibrin-binding affinity. The main biological proteins that have been mimicked include fibrin knobs, human plasminogen, anti-plasminogen, and fibronectin's fibrin-binding domain.

Fibrin Knobs:

The amino acid sequences of the N-terminal knobs 'A' and knobs 'B' are GPRV and GHRP, respectively[52], though mostly the knob 'A' has been pursued as a mimic because its binding

affinity is higher than that of the knob 'B' at both the 'a' and 'b' polymerization holes. While GPR is the shortest amino acid sequence possessing affinity for fibrin, it was shown that the sequences GPRP, to a greater extent GPRPP, and to an even greater extent GPRPFP possess more stability, can bind fibrin even more strongly, and are more resistant to proteolysis[53-56]. Variations of fibrin knob 'A' mimics have been utilized to alter fibrin architecture[57, 58], incorporate bio-molecules into fibrin matrices[16], inhibit the formation of clots[59], and provide contrast for CVD diagnostic imaging[31, 55, 56, 60].

Human Plasminogen:

The five kringle domains found at the N-terminal region of human plasminogen are responsible for its ability to bind fibrin with high specificity, and of these domains, kringle-1 has been shown to possess the strongest individual specificity with a K_a of 60mM, which is just slightly less than the 86mM K_a of native plasminogen[12, 61, 62]. However, the molecular weight of the Kringle-1 domain, at 10kDa is much more manageable than plasminogen's at 88kDa[61]. Kringle-1 has been successfully employed in the targeted delivery of thrombolytics[12] and as a method of prolonging growth factor retention in wound matrices to improve neovascularization and overall healing in rat models[63].

Antiplasmin:

The enzyme α2-antiplasmin serves as one of the body's primary inhibitors of plasmin-mediated fibrinolysis, and an ability to become covalently crosslinked into the fibrin matrix by FXIIIa greatly enhances this role[64, 65]. The 1.6kDa α2-AP peptide with amino acid sequence N13QEQVSPLTLLK24 is only a fraction the size of native anti-plasmin's 70kDa, though it maintains a strong fibrin-binding ability. α2-AP's time-table for incorporation into fibrin clots mimics the enzymatic activity of FXIIIa with a half-life of about 20 minutes and virtually undetectable activity after an hour[64]. The peptide has been used in a preliminary study to detect blood clots in mice, though predictably, it was only effective in detecting very fresh clots [65]. Additionally, the α2-AP peptide has been used to covalently link growth factors to fibrin scaffolds to prolong their retention and bioavailability to infiltrating cells and thus enhancing angiogenesis[66].

Fibronectin Fibrin-Binding Domains (FFBD)

Fibronectin is a glycoprotein present in the blood and extracellular matrix that has functional roles in binding to fibrin, cells, heparin, and other biological species[67]. It is known that its N-terminus possesses a fibrin-binding site composed of several molecular fingers that can be covalently cross-linked into fibrin via FXIIIa[68]. Furthermore it is possible to isolate a small portion of this binding domain without a severe reduction in fibrin-binding affinity in comparison to native fibronectin; K_d's of the 25.9kDa fragment and 220kDa full-length proteins are 18nM and 2.1nM, respectively[69]. Peptides mimicking two, three, or five-fingers of this sequence have been synthesized explicitly for CVD imaging applications though the focus has been on the 12kDa 2-fingered peptide version that possesses the most favorable pharmacokinetics[67, 70]. Early development showed that that the 2-fingered peptide bound optimally to fibrin in the presence of factor XIIIa with significantly less incorporation into clots more than a day old[70] and a decreased ability to positively identify DVT older than a week in human subjects[67]. Phase II clinical trials in humans showed promising diagnostic sensitivity[47] (Table 1).

The Evolutionary Approach

The "Evolutionary Approach" truly represents the second generation of fibrin-targeting in that through its use of phage-displayed biopanning, it completely bypasses the use of animals to create fully human antigen-binding species in a high-throughput manner. While other approaches may utilize phage biopanning as part of their refinement of fibrin-binding entities, the "Evolutionary Approach" is the only strategy that is not copying what nature has already produced or relying upon the immune systems of non-human species to generate the initial novel binding moiety.

The diversity of biopanning is rooted in fundamental immunology principals. Whereas the human immune system relies upon a multitude of V gene arrangements to achieve diverse antigen-recognition capabilities, phage biopanning includes a similarly wide variety of V gene arrangements within phage libraries designed *in vitro*[71]. Once infected into bacteria, the various proteins or peptides encoded by these genes are expressed as fusions with phage coat proteins and displayed at the bacterial surface[72]. Selection of specific binders involves panning these fusion peptides against immobilized antigen, washing away any weak-binding or non-binding entities, and then eluting and re-amplifying the binders via re-infection of *E. coli*. Competing binding species, such as fibrinogen or soluble plasma proteins, may easily be introduced during the panning stage as a method to eliminate unwanted cross-reactivity in peptide candidates. Several rounds of such panning and bacterial re-infection may be required until a significant enrichment of antigen-specific phage can be achieved[73]. Importantly, V genes are subject to random mutation during the re-infection phase, and to encourage this, mutator strains of bacteria, or alternatively, error prone polymerase, may be utilized in between panning steps in attempt to maximize binding affinity through each successive phase of panning[71].

Tumor Bio-Panning:

The CREKA peptide was discovered through selection of phages capable of homing to tumor sites *in vivo* within MMTV-PyMT transgenic breast cancer mice[74]. While the exact site of binding is not known, CREKA is strongly suspected to interact with fibrin-fibronectin complexes due to the facts that it has been shown to co-localize with anti-fibrinogen antibodies in atherosclerotic plaques[11], it will not bind tumors in fibrinogen-null mice or fibronectin-null mice [74, 75], and it possesses a proven ability to induce localized tumor clotting[74]. This latter characteristic creates additional binding sites for the peptide itself, thus constituting a self-amplification functionality[75]. Notably, anticoagulation therapy does not significantly affect its performance. Though the peptide's specific binding affinity has not been reported, it has shown promise for its ability to deliver thrombolytics to clots[11] and to target gliomas[76], prostate cancers[75], atherosclerotic plaques[11, 77], and lung cancer[78].

Clotted Plasma Bio-Panning:

The CLT-1 peptide with the sequence CGLIIQKNEC is another entity from the Ruoslathi group that targets fibrin-fibronectin complexes, though it was originally panned against clotted plasma in the presence of liquid plasma[79]. Its primary target is actually thought to be fibronectin, since it will home to tumors to a minimal extent in fibrinogen-null mice but not at all in fibronectin-null mice. CLT-1 has successfully been conjugated to gadolinium for magnetic imaging of liver fibrosis[80], cancer[81], and atherosclerotic plaques[82]. Its specific binding affinity has not been reported.

Knob 'A' Bio-Panning:

The creators of the AP2 fibrin-binding entity[35] epitomized the potential of fibrin-biopanning by performing two successive rounds of panning separated by a round of enhancing molecular evolution in between. The first round of biopanning utilized a large combinatorial library against the first ten residues of immobilized fibrin knob 'A' and identified an scFv, AP1, capable of specifically binding fibrin with an affinity of K_D = 7μM. A new 13-billion clone library based on the important residues of AP1 was subsequently created utilizing partially degenerate oligonucleotides in a PCR-assembly procedure. This novel library was then panned against fibrin 'A' knob to produce a monomeric scFv, AP2, with a significantly enhanced affinity for fibrin of K_D = 44nM. Impressively, AP2 was also able to be efficiently expressed as a full-length IgG antibody or a $F(ab')_2$, therefore lending the potential for a range of pharmacokinetics. Importantly, there is no detectable binding of AP2 to fibrinogen, and in its initial study of efficacy, it was able to home to fibrin-rich F9 murine teratocarcinomas *in vivo*. Accumulation in SKRC-52 tumors, however was not significantly higher than in that of non-specific uptake in the heart, spleen, and liver, most likely indicating that knobs 'A' are available in varying amounts across tumor types.

DD(E) Complex and Fibrin Panning:

Alternate rounds of panning against polymerized fibrin and immobilized DD(E) complexes were utilized to isolate three related classes of small cyclic peptides referred to as Tn6, Tn7, and Tn10. Each round of panning was preceded by phage incubation with fibrinogen to eliminate non-specific binders. As intended, these peptides all bind to DD(E) complexes with similar affinity to that of fibrin – in the low micromolar range, and they bind to fibrinogen with at least 100-fold weaker affinity than that for fibrin. Tn7 and Tn10 share a binding site unique from Tn6 that is thought to play a role in fibrin polymerization since both Tn7/10 possess the ability to incite fibrinogen self-association[29]. Both Tn6 and Tn7 peptides have been modified for conjugation to four magnetic resonance reporter molecules without a significant loss in fibrin affinity[83-86]. A version of the modified Tn6 peptide, designated EP-2104R, has been most rigorously tested up through phase II clinical trials. Human testing revealed blood-pool background signal to be a significant problem at early time points, therefore requiring a separate evaluation of sensitivities for scans conducted at least two hours post-injection that greatly improved sensitivity (Table 1)[46]. Subsequent versions of EP-2104R as well as a novel cyclic peptide a with similar fibrin-binding active site, called FibPep,[87] have been developed and conjugated to PET and SPECT reporters under a variety of labels and have shown promise for detecting thrombi in animal models[30, 88-90].

Whole-Fibrin Bio-Panning

Only two attempts have been made utilizing phage biopanning against three-dimensional polymerized fibrin. The first utilized non cross-linked fibrin and isolated a scFv whose fibrin-binding was not extensively characterized, though it appears fibrin affinity was not very strong, exhibiting only a six-to-one preference over fibrinogen[91]. The second effort utilized cross-linked fibrin and also introduced competition to the panning step with soluble fibrinogen. This ultimately identified several fibrin-specific scFv and sdFv binders, the best designated H6, which possesses a binding affinity for fibrin in the nanomolar range[92]. This sdFv was found to be capable of binding to fibrinogen very weakly, which implies that its targeting site exists within

the fibrinogen precursor molecule and that conformational changes associated with the conversion of fibrinogen to fibrin render it more accessible. The fact that H6 was panned against fully-polymerized cross-linked clots implies that its fibrin-targeting capabilities should not be limited by the clot age. In its debut study, multiple copies of H6 were conjugated to microgels in order to form synthetic platelet-like particles capable of reducing bleeding times in rats on par with the clinical standard, FVIII, and faster than that of infused fresh platelets[92].

Table 2

Binding Molecule	Affinity	Molecular Weight (Da)	Molecule Type	Ligand	References
Evolutionary Approach					
H6	199nM	17,492	sdFv	Crosslinked Fibrin Clot	[92]
SP2	44nM	45,570 (scFv)	scFv, mAb, or $F(ab')_2$	Knob 'A'	[35]
Tn6	4.1uM	1397	peptide	DD(E) Complex/Fibrin	[29, 30, 46, 84, 85, 88, 90]
Tn7	4uM	1262	peptide	DD(E) Complex/Fibrin	[29]
Tn10	8.7uM	1868	peptide	DD(E) Complex/Fibrin	[29]
FibPep	800nM	2738	peptide	DD(E) Complex/Fibrin	[30, 87, 89]
CLT-1	Not Reported	1120	peptide	Fibrin-fibronectin complexes	[79-82]
CREKA	Not Reported	606	peptide	Fibrin-fibronectin complexes	[11, 74-78]
Yan ScFv	~6:1 fibrin preference	27,000	scFv	Fibrin Clot	[91]
Biological Mimicry Approach					
GPRPFPAC	25 uM	844	peptide	Holes 'a' and 'b'	[16, 53-59]
Fibronection N-terminal FBD	18nM	25,000	peptide	Fibronectin-binding domain	[68, 69]
Kringle-1	12-17 uM	10,000	peptide	Plasminogen binding-domain	[12, 61-63]
α2-AP	Covalent Bond	1600	peptide	Anti-plasminogen binding domain	[64, 65]
Shotgun Approach					
MH-1	0.67nM	~150,000	mAb	Freeze fractured fibrin, DD-Dimer	[49]
NIH 1H10	3.9nM	96,000	mAb	Freeze-fractured fibrin, E Region	[48]
NIB 5F3	4.3nM	~150,000	mAb	E-Fragment	[48]
102-10 mAb	Not Reported	~150,000	mAb	Bβ (149-234)	[50]
A Priori Approach					
DI3B6/22-80B3, ThromboView	1.6nM	~55,000-110,000	$F(ab')_2$ or Fab'	DD-Dimer	[41, 42]
Anti-Fgn 17	Not Reported	~150,000	mAb	Knob 'A' (GPRVVE)	[36]
T2G1	Not Reported	50,000 – 150,000	mAb or Fab'	Knob 'B'	[22, 25]
59D8 Centocor C22A	Not Reported	36,000 – 150,000	mAb, Fab or scFv	Knob 'B'	[10, 17, 24, 26]

Table 2: An overview of the various distinct fibrin-binding entities and their respective characteristics. Molecular weights were estimated based on antibody fragment type in cases where precise molecular weights were not reported. Affinity measurements represent the most recent values recorded to date. The methods and accuracy associated with each measurement may vary.

Summary and Future Outlook:

There exists a wide variety of strategies for specifically targeting different fibrin epitopes from particular fibrin domains to complicated multi-component three-dimensional fibrin complexes. An impressive amount of research has been conducted on fundamental characteristics of fibrin systems, their roles in an assortment of pathologies, and their potential use in diverse engineering applications. This information can potentially be utilized to tailor particular fibrin-targeting strategies—with all their distinct advantages and disadvantages— to ultimately achieve successful clinical translation. The strategies within the "Evolutionary Approach", especially, are high-throughput, provide the capacity to choose a desired physical platform, and offer the largest degree of application-specific customization to date. Many targeting entities isolated through this latter approach represent recent advances that have not yet been fully tested in larger animal models, let alone humans. Nevertheless, they possess great potential for the breadth of both current and future biomedical engineering challenges.

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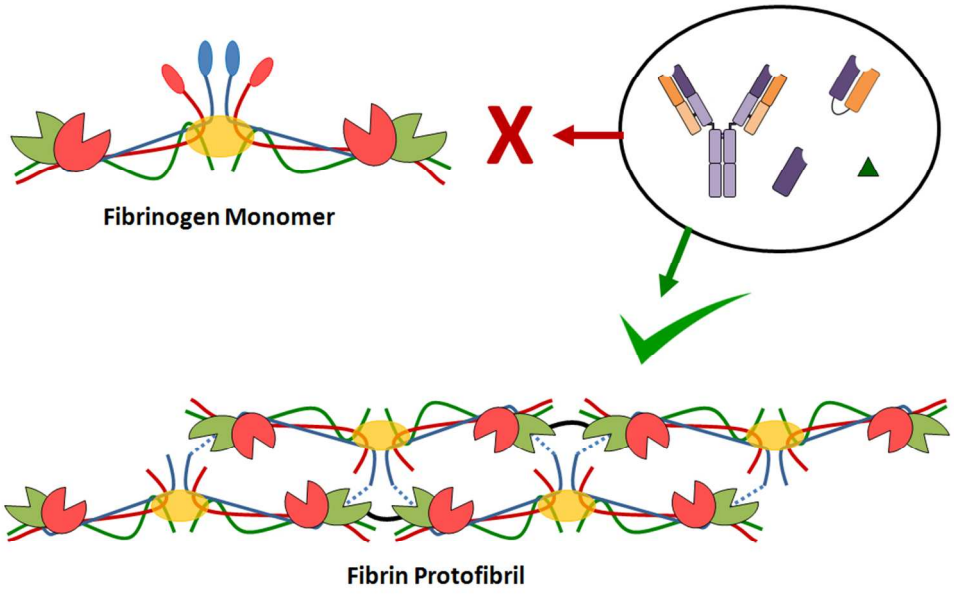

A variety of antibodies, antibody fragments, and small peptides have been engineered to specifically target fibrin over its precursor fibrinogen.