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Fibrin Matrices as (Injectable) Biomaterials: Formation, Clinical Use, and Molecular Engineering

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This review focuses on fibrin, starting from biological mechanisms (its production from fibrinogen and its enzymatic degradation), through its use as a medical device and as a biomaterial, and finally discussing the techniques used to add biological functions and/or improve its mechanical performance through its molecular engineering. Fibrin is a material of biological (human, and even patient's own) origin, injectable, adhesive, and remodellable by cells; further, it is nature's most common choice for an in situ forming, provisional matrix. Its widespread use in the clinic and in research is therefore completely unsurprising. There are, however, areas where its biomedical performance can be improved, namely achieving a better control over mechanical properties (and possibly higher modulus), slowing down degradation or incorporating cell-instructive functions (e.g., controlled delivery of growth factors). The authors here specifically review the efforts made in the last 20 years to achieve these aims via biomimetic reactions or self-assembly, as much via formation of hybrid materials.

1. Introduction

Fibrin is an enzymatically processed, fibrillary assembled derivative of fibrinogen.[1] Importantly, while it is easy to provide a molecular definition of fibrinogen (see Section 2.1 "Fibrinogen and its Conversion into Fibrin"), fibrin is considerably more elusive: under this name we may refer to networks where fibers a) have different sizes, b) have different topologies, and c) may self-assemble predominantly through affinity interactions, or be chemically cross-linked. In short, using the word "fibrin" we do not refer to a characterized molecular entity, but rather to a material with a well-defined biochemical process of production.

In nature, fibrin plays predominantly a dual role: as an hemostatic agent, since its formation allows to stop bleeding; and as a provisional, remodelable matrix that is reprocessed during both healing or fibrotic processes. It is worth recalling that fibrin neither is a blood clot, nor is a major component (in weight) of a blood clot; it is, however, the component that holds it together, as a sort of adhesive fisherman's net full of cells and colloids.

The possibility to artificially replicate the coagulation cascade in a controlled manner makes it possible to form materials in situ, and to do at virtually any accessible location using injectable precursors; further, as their natural counterparts, fibrin-based materials in a first instance can act as hemostatic agents, sealants, and tissue adhesives, and in a second instance they would support and to a certain degree instruct healing processes.

Here, we aim to provide an updated overview of fibrin in its roles as a natural and as an artificial biomaterial.

2. Fibrin as a Natural Material

2.1. Fibrinogen and Its Conversion into Fibrin

2.1.1. Fibrinogen Molecular Structure

Fibrinogen is a 340 kDa homodimeric protein, whose quaternary structure vaguely recalls the shape of a dumbbell; the peripheral areas—where weight plates would be placed in a dumbbell—have a globular conformation and are referred to as D regions (**Figure 1**, top), whereas another globular area is located in the center, and is referred to as the E region. Connecting the D and E regions there are straight segments with a triple-helical coiled-coil conformation.

The two symmetric halves are each composed by three independent protein chains—the Aα (610 aa; 66.5 kDa), Bβ (461 aa; 52 kDa), and γ chains (411 aa; 46.5 kDa)—and the

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overall fibrinogen quaternary structure heavily relies on a large number of sulfur-based structures, namely 29 disulfides interconnecting them,[2] but also >60 hydrophobically associating methionines[3]; the oxidation of the latter, in particular the γ-78, β-367, and α-476 residues, produces weaker fibrin, with thinner fibers and slower fibrinolysis.[4]

For what attains to disulfides, it is useful to distinguish them in three groups: 1) three central disulfides critical for keeping together the two fibrinogen halves (one Aα-Aα' and two γ-γ'[5]), which together with additional four disulfide per half (two Aα-Bβ, one Aα-γ, and one Bβ-γ) compose the "N-terminal disulfide knot" in the E region, where indeed the N-termini of all chains are located. Please note that the Aα C termini (aka αC domains) fold back nearby, but they are typically not considered part of the E region. 2) In each half, six disulfides interconnecting the three chains and stabilizing the triple-helical coiled-coil regions (two Aα-Bβ and three Bβ-γ). 3) In each half, three intra-chain (one per chain) disulfides located within hydrophobic domains of the D regions.

2.1.2. Fibrinogen Production

Fibrinogen is primarily synthesized by hepatocytes in the liver, at a rhythm of a few grams per day.[6] Once secreted in the blood stream, typically it reaches a concentration ranging between 1.5 and 4.5 mg mL^{-1}[7] with a half-life of ≈4 days.[8] Fibrinogen expression and secretion in the blood stream typically increases under inflammatory conditions: raised fibrinogen levels (and lower albumin) are recorded, for example, in chronic obstructive pulmonary disease,[9] diabetes,[10] in smokers,[11] and more generally in the age-related pro-inflammatory state[12]; there is an established association between the circulating levels of inflammatory cytokines such as interleukin-6 (IL-6) and those of fibrinogen,[11–13] which is possibly due to fibrinogen synthesis promoters containing IL-6-responsive elements.[14]

2.1.3. Fibrin Formation (Blood Clotting)

In the formation of a clot, multiple (mostly cellular) elements are "jammed" together by a network of fibers. From a materials science point of view, the key step of this process is the conversion of soluble macromolecules (fibrinogen) into network precursors ready for self-assembly, that is, fibrin (Figure 1). At a molecular level, this conversion is based on chemical changes operated by a serine protease (thrombin) in the central E region of fibrinogen; this region—as mentioned—contains the N-termini of the six polypeptide chains, four of which (the two Aα and the two Bβ) feature thrombin-cleavable sites close to their N-termini. Thrombin is produced from a circulating precursor (prothrombin) through a complex cascade of activation mechanisms, usually grouped in the intrinsic and in the extrinsic pathways; we address the reader to specific reviews to further elaborate in this area.[15,16] Thrombin cleaves an arginine-glycine bond and releases a sequence from Aα (fibrinopeptide A; in humans ADSGEGDFLAEGGGV**R**(-**G**PRV...)) and one from Bβ (fibrinopeptide B; in humans: GVNDNEEGFFSA**R**(-**G**HR...)),[17] uncovering new motifs referred to as A knobs (GPRV) and B knobs (GHR), respectively at the α and β chain N-termini. They can subsequently bind to complementary sequences—referred to as a and b holes—located, respectively on the γC and βC nodes in D regions of other fibrin(ogen) molecules.[18] a:A knob–hole interactions occur immediately after cleavage and are the main driving force for the association of fibrinogen into half-staggered, double-stranded, and rod-like oligomers that grow into protofibrils; the latter exhibit a typical periodicity of 22.5 nm.[19] It is critical that fibrinopeptide A is cleaved before fibrinopeptide B for effective b:B interactions to occur;[20,21] the latter have an affinity about six times lower than the a:A coupling (K_d = 140 μM vs K_d = 25 μM),[22,23] and are thought to play a role in fibril lateral aggregation (thicker fibers when a:A interactions are weakened by selective mutations[24]), which becomes important after they reach a critical length of 600–800 nm.[23] The lateral growth is largely irregular and the fibers become progressively less dense and softer the larger they grow, with density and stiffness both scaling with diameter D as $D^{-1.6}$;[25] the general model therefore is of fibers containing a dense, highly interconnected core with a relatively sparse periphery where both the protein density and inter-fibril bond density decrease as $D^{-0.6}$ and $D^{-1.5}$, respectively.[26] Competing with lateral aggregation,

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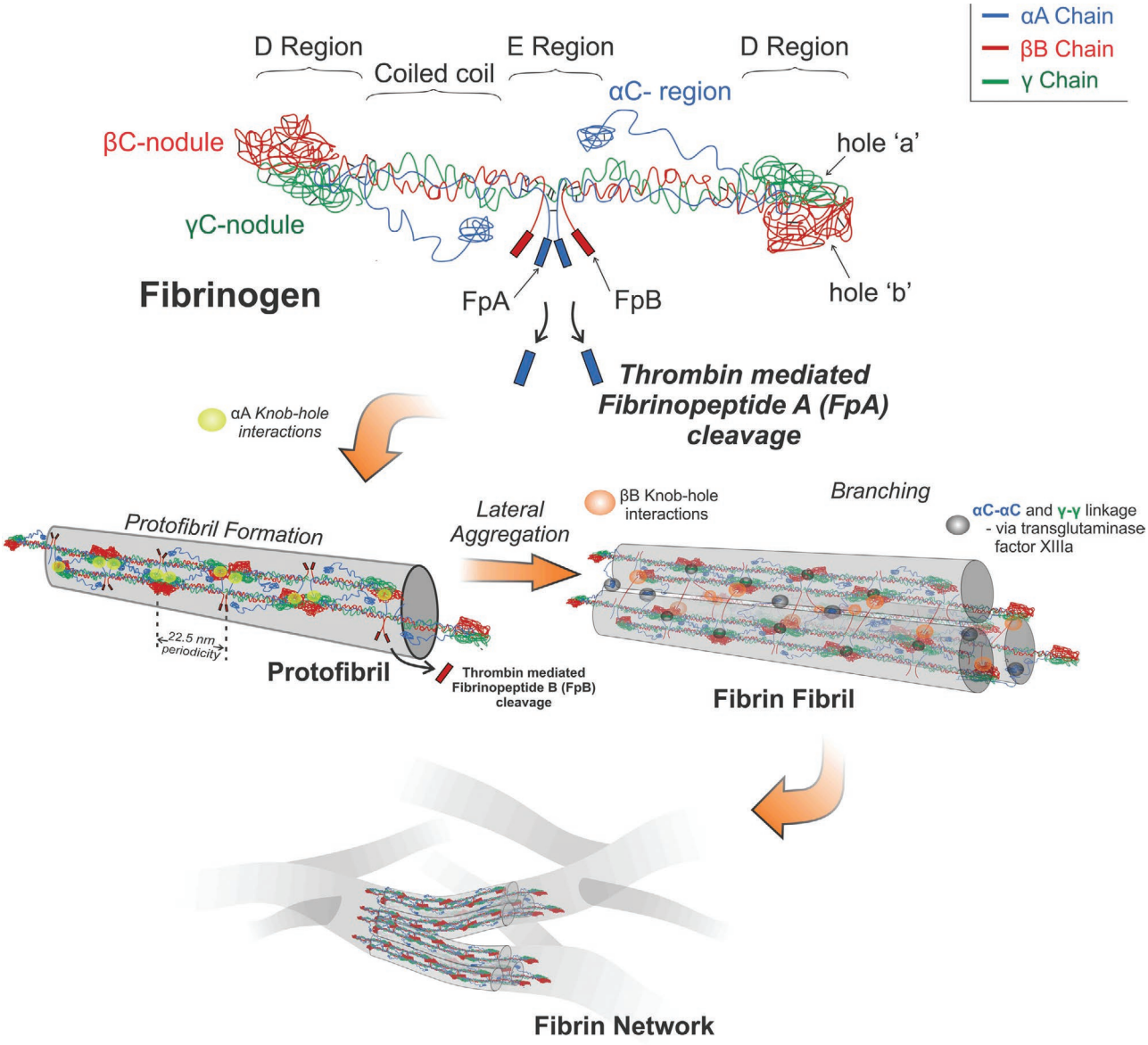

Figure 1. The formation of fibrin from fibrinogen. A fibrinogen molecule consists of six polypeptide chains interlinked by disulphide bonds to form a symmetric molecule with outer D domains consisting of βC and γC nodules and a central E domain which are connected by coiled-coil regions. The thrombin mediated cleavage of fibrinopeptides A lead to the formation of protofibrils via αA knob–hole interactions. This is followed by the slower process of fibrinopeptide B cleavage by thrombin which leads to the lateral aggregation of these protofibrils. These fibrils branch and interconnect to form a fibrin network. A transglutaminase enzyme, typically referred to as Factor XIII in the coagulation cascade, stabilizes the network by producing αC–αC and γ–γ linkages (in the form of glutamin–lysine couplings) between adjacent fibrils and fibers.

fibers may also undergo branching, typically in two forms: 1) bilateral branching involves the separation and divergence of two protofibrils due to incomplete lateral aggregation; 2) trimolecular junctions form from a terminating fibrin monomer with only 1 A:a bond growing its own independent strand.[1]

The fibers are further stabilized and made more compact by interactions between αC domains in adjacent fibers; important, these interactions are not necessary for fiber formation, but make them thicker with fewer branching points.[27] This has some pathological implications; for example, liver cirrhosis is known to induce over-carbonylation of the α chain,[28] leads to more dense clots with thin fibers,[29,30] possibly due to the disruption of αC interactions in lateral aggregation.[28] Finally, the network undergoes a chemical cross-linking that hardens them[31] and stabilizes them against proteolytic degradation[32]; Factor XIIIa (a transglutaminase whose zymogen – Factor XII – is activated by thrombin and calcium[33]) introduces covalent ε(γ-glutamyl)lysyl cross-links between α-α, as well as γ-γ and α-γ chains in adjacent fibrils.

The fibrin clot formation can be influenced by both post-transcriptional and post-translational events. For example, in healthy individuals 8–15% of fibrinogen contain a splice variant called γ' differing in the C-terminus, 1% of fibrinogen molecules being γ'/γ' homodimers;[34,35] this polymorphism leads

to thinner fibers and a mechanically weaker network,[34,36,37] which appears to increase the risk for myocardial infarction.[38] The fibrinogen molecule also has four sites available to interact with carbohydrates[39] and the N-glycosylation appears to have a role in the regulation of lateral aggregation.

It is worth noting that thrombin is not the only enzyme capable of triggering the transformation of fibrinogen into a form of fibrin. For example, snake venoms can have a strong procoagulant activity, and, for example, the eastern diamond-back rattle snake's venom contains thrombin-like crotalase enzymes which selectively cleave fibrinopeptide A.[40] This point, however, should not be generalized because a range of snake venoms have exactly the opposite activity, and contain anti-thrombotic elements including disintegrins (inhibitors of platelet aggregation), specific inhibitors of steps of the coagulation cascade, as well as fibrinolytic agents.[41,42] Ancrod (from the venom of Malayan pit viper) is somehow a special case: it cleaves fibrinopeptides A only (fibrin clots with no b:B interaction), but partially degrades α chains,[43] and may take part in other degradative processes, so that its defibrinogenic rather than thrombogenic activity has made it a candidate for ischemic stroke therapy.[44]

2.2. Fibrin Degradation (Fibrinolysis)

Fibrinolysis allows the reversal of clotting, primarily through the proteolytic action of plasmin; it is worth pointing out that fibrin formation and fibrinolysis are coordinated processes, which allows the maintenance of blood fluidity and at the same time to prevent blood loss. However, fibrinolysis not only reduces the chances of thrombosis, but also allows remodeling of a provisional matrix into a more or less functional tissue; these fibrinolytic processes may also be performed by enzymes other than plasmin, such as various matrix metalloproteinases (MMPs) such as membrane-type 1 MMP (MT-1-MMP aka MMP-14),[45,46] stromelysin 1 (MMP-3),[46,47] and matrilysin (MMP-7),[46] with mechanisms that differ in soluble fibrinogen and in cross-linked fibrin and that release different fragments (but most often containing (parts of) the D regions). The release of these fragments contributes to instructing the healing processes: for example, the release of E and D region-containing fragments can stimulate neutrophil chemotaxis[48] and angiogenesis.[49,50]

Here, however, we focus predominantly on plasmin-mediated fibrinolysis; plasmin can cleave fibrin at up to 34 sites, which are mostly placed in the coiled-coil and in αC regions (areas B and C in **Figure 2**A).[51] Plasmin is produced when plasminogen is activated by tissue type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), both of them activated by plasmin itself in a positive feedback system[52] (Figure 2B). Conformational changes during the conversion of fibrinogen to fibrin allow the binding and colocalization of tPA and plasminogen predominantly onto lysine residues at the αC domain of fibrin (inset B in Figure 2A), with additional binding for both tPA and plasminogen in the D region (inset A in Figure 2A). The proximal localization allows the production of most plasmin directly on fibrin (tPA alone is a weak activator), which means that fibrin can positively regulate its own degradation. Also plasmin also plays a major positive feedback role in fibrin degradation by contributing to tPA and uPA activation (Figure 2B), and by exposing C-terminal lysines for the further attachment of tPA and plasminogen (inset C in Figure 2A). Note that the binding to these newly exposed C-terminal lysines is typically stronger ($K_D \approx 10^{-8}$ M) than that to the original intrachain binding ($K_D \approx 10^{-5}$–10^{-6} M), as intact fibrin contains no C-terminal lysines.[53] Also a number of other factors can contribute to plasmin activation (Figure 2B), for example, the bacteria-derived streptokinase and staphylokinase,[54] which have been clinically employed in fibrinolytic therapies for the treatment of thrombosis, although with concerns about the risk of hemorrhage.[55] Another clinically evaluated fibrinolytic agent is desmoteplase (derived from vampire bat saliva), which shows a highly selective fibrin-bound activation of plasminogen, thereby avoiding the problematic systemic activation of plasmin.[56] In this mechanism of locally enhanced plasmin activation, cleavage events predominantly occur transversally rather than longitudinally in the fibers.[57] At an individual fiber level, thinner fibers reportedly lyze quickly, whereas fibers with diameters exceeding 200 nm would appear to elongate during lysis more than being chopped[58]; this is interpreted as a relaxation from strain introduced during fiber assembly, which may hinder the binding plasminogen or of its activators, in agreement with the increased resistance of stretched fibrin to fibrinolysis.[59] However, at a whole clot level, the situation is different and dense clots made of thinner fibers generally degrade slower[60]; individual thin fibers may degrade more rapidly,[61] but networks made of larger fibers are more porous and allow better diffusion of fibrinolytic agents (Figure 2C). It has also been shown that fibers also tend to aggregate during the degradation process.[62] Fibrinolysis can be inhibted through several mechanisms[53]: a) lysine analogs (ε-aminocaproic acid (ACA) or tranexamic acid) compete with lysine residues in fibrin, thereby reducing plasminogen activation; their low cost and safe profile makes them popular drugs, for example, to reduce blood loss in myocardial infarction.[64] b) The mode of action of thrombin activatable fibrinolysis inhibitor (TAFI) is not much different, since they reduce tPA/plasminogen binding to fibrin by cleaving lysine sites in the latter. c) Finally, inhibitors such as circulating plasminogen activator inhibitor-1 (PAI-1) or α2-antiplasmin (α_2PI) act predominantly on circulating targets, but much less on fibrin-bound enzymes. d) the serine proteinase aprotinin, on the contrary, acts on plasmin independently on its bound state[65]; it has been widely applied in the clinic, but it significantly increases the risk of death (e.g., 13% to 30% 5-year mortality after coronary bypass surgery[66]).

We refer the reader to more extensive reviews for a more detailed analysis of the process of fibrinolysis.[51,53,67]

2.3. Mechanical Properties and Morphology

Fibrin is a soft material, whose stiffness values can significantly overlap with those of most human tissues, with the notable exceptions of bone and fibrocartilage. A blood clot typically has a Young's modulus around 1 kPa[68] and a shear modulus of a few hundreds Pa[69]; platelet-rich fibrin is very similar, for example, exhibiting a tensile elastic modulus of about 1 kPa with 3 mg mL^{-1} fibrinogen.[70] At higher concentrations, for example, in fibrin glues, elastic moduli increase considerably;

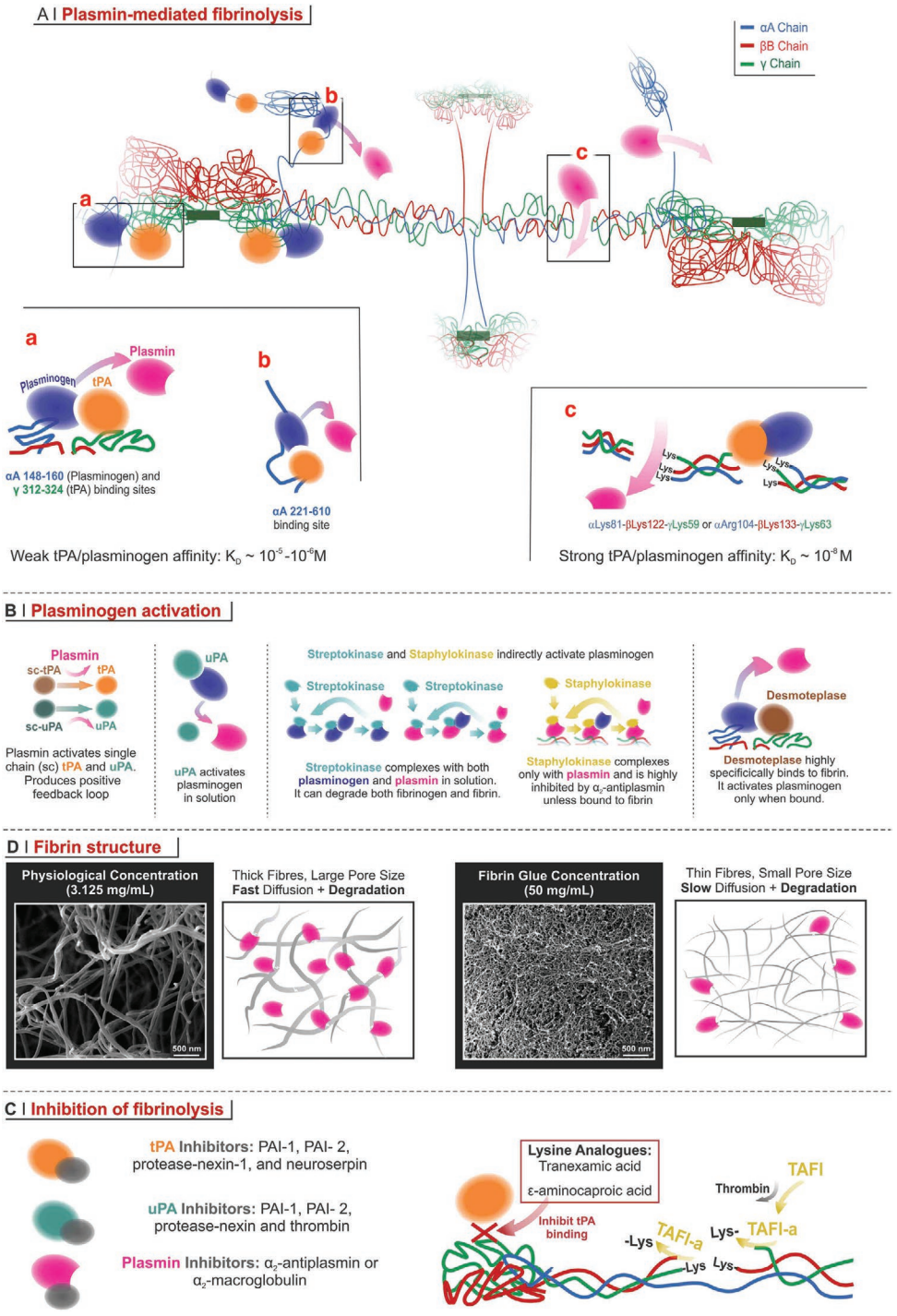

Figure 2. A) tPA and plasminogen bind weakly on the αC chain and at specific sites in the D region (insets a and b) and there produce plasmin. Plasmin then cleaves sites in the coiled-coil region, exposing C-terminal lysines that further bind tPA and plasminogen (inset c), leading to a positive feedback. B) *Left to right*: single-chain (sc) tPa and uPA are activated by plasmin. Unlike tPa, uPa does not bind to fibrin and acts in solution. Streptokinase and staphylokinase activate plasminogen, the first by binding to both plasminogen and plasmin to activate other plasminogen molecules in solution, the second only binding to plasmin bound to fibrin (as it is strongly inhibited by α_2-antiplasmin when in solution). Desmoteplase mimics the mechanism of tPA, but is more selective, only activating plasminogen when bound.[56] C) SEM images and sketches of fibrin networks; at a few mg mL^{-1} (physiological), they have thicker fibers and a larger pore size, allowing faster plasmin diffusion and degradation. At higher concentrations (fibrin glues) fibrin networks are denser with smaller fibers, delaying both diffusion and degradation.[63] D) Inhibitors of fibrinolysis include plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) and nexin, which act on both tPA and uPA. Also neuroserpin and thrombin can be added to this class, although respectively acting only on tPA or on uPA. α_2-antiplasmin and α_2-mactoglobulin bind and inhibit plasmin. Finally, lysines (exogenous: tranexamic acid,ε-aminocaproic acid; or generated by TAFI) inhibit tPA binding to fibrin.

Table 1. Effects of controlling factors on fibrin properties.

Factor		Structural outcome	Ref.
Fibrinogen conc.	↑	Thinner but more dense fibers, higher modulus	[76,77]
Thrombin conc.	↑	As above but to a lesser degree	[78]
Calcium conc.	↑	Higher rate of fibrin monomer production, leading to thicker and longer fibers with lower branching. The storage modulus of the clot peaks at ≈1.5 mM $CaCl_2$	[79]
Fibrinogen oxidation	↑	Generally, inhibited thrombin-triggered gelation, often but not always faster degradation	[80–82]
	Methionine oxid.	Thinner but more dense fibers, lower stiffness. Less degradation	[4,80]
	Histidine oxid.	Reduced Aa association. Unreported effects on gels, but likely lower modulus	[83]
Ionic Strength	↑	Disruption of protofibril lateral aggregation leading to thinner fibers. High HEPES concentration significantly decreases permeability but not the shear modulus	[84]
Flow	↑	Fiber alignment, increased shear modulus	[85–87]

for example, an elastic modulus of about 100 kPa has been recorded for Tisseel.[71]

At concentrations below 1 mg mL^{-1}, moduli as low as a few tens of Pa have been recorded.[72] This very soft character can have a significant effect on the phenotype of cells cultured in or on it, for example, decreasing the stiffness of cancer cells and increasing the tumorigenic/stem-cell-like character,[73] or selecting neuronal versus glial differentiation when mechanically similar to brain stiffness (a few hundreds Pa[74]).

It must be noted, however, that literature data are affected by a significant variability; for example, a tensile modulus of 28 kPa has been reported for 2 mg mL^{-1} fibrin.[75] Morphological, and as a result also mechanical properties strongly depend on the details of fibrillar self-assembly. In turn, the latter are controlled by variables, including fibrinogen, thrombin, and calcium concentration, the ionic strength and composition, as much as the possibility of fibrinogen chemical modification, for example, its oxidation. **Table 1** summarizes some of the most important effects.

It is worth pointing out, however, that although we mostly list thermodynamic variables (e.g., the concentration of reagents), fibrin properties are in reality dominated by various forms of kinetic control. For example, a higher fibrinogen concentration should produce thicker fibers because of accelerated lateral growth; on the contrary, due to strongly limited diffusion in a growing network, the more concentrated fibrin is, the thinner (and more numerous) are its fibers.[76] Similarly, one would expect that larger fibers are stiffer, but—as previously mentioned—their modulus in reality decreases with their diameter as $D^{-1.6}$.[25] A last word of warning is that in a "real life" situation, several controlling factors act simultaneously, and it is hardly possible to foresee the final result. For example, fibrin clots from stroke patients show thinner fibers (30 nm average diameter) than those in healthy individuals (90 nm),[88] and their clots are less porous and less degradable[89] (likely due to poorer diffusion of enzymes). Since the concentration if fibrinogen is basically constant, this effect may be to ascribe to higher levels of thrombin or to its quicker activation.

An important mechanical feature of fibrin gels is the combination of linear elasticity at low strain (and short times) with its stiffening at higher strains. Strain stiffening is a very remarkable effect (moduli may increase up to 20 times); albeit to a lower extent, it can be seen also in the presence of platelets in plasma clots,[90] and is shown down to the level of individual fibers.[91] This phenomenon has been interpreted as due to fibers behaving as tight protofibril bundles at low strain, and loose assemblies of almost independent protofibrils at higher strains,[92] although fiber alignment[72] and non-affine deformation mechanisms[93,94] are almost surely additional contributors. At a supramolecular scale, simulations have shown that high strain may cause an α-helix to β-sheet transition in the coiled-coil regions between E and D domains, at least for extensions above 40 nm (or forces of 175 pN);[95] experimentally an α-helix to β-sheet transition has indeed been observed via infrared spectroscopy.[96] Other unfolding transitions in the γ domains[95] and the αC regions[97,98] are also thought to contribute to fibrin high extensibility and possibly modulus increase (i.e., stiffer chains). Some of these transitions are not necessarily irreversible: fibrin can sustain a 2.8-fold extension without permanent lengthening, and a 4.3-fold extension prior to rupturing.[91]

As side effects of these unfolding transitions, high strains lead to fiber aggregation and water expulsion, thereby providing additional stiffening contributions.[99] This, however, leads also to negative compressibility:[77,99,100] fibrin typically exhibits a Poisson's ratio above 0.5, which means that it shrinks upon deformation; this is ascribed to denaturation of fibres coupled with the expulsion of water.[99]

It is worth mentioning that a fibrin matrix can be hardened by cells dispersed within, with up to a threefold increase in stiffness; this has been ascribed to cells preferentially adhering to the "floppy" modes (non-elastically active regions) within the network rather than to an axial stretching of the fibres themselves.[101]

2.4. Biophysical Properties

Fibrin is a cell-remodelable matrix. In order for cells to be able to remodel it, fibrin biophysics must be able to support their adhesion and instruct them in their migratory and degradative activity.

These actions can be a result of cells directly interacting with fibrin; for example, platelets adhere through the high fibrin affinity of $\alpha_{IIb}\beta_3$ integrin[102] and fibroblasts directly bind to

fibrin fibers through $\alpha_{(v)}\beta_3$ binding to exposed RGD sites.[103] Inflammatory cells such as neutrophils can adhere to fibrin through a mix of interactions involving P-selectin and integrins,[104,105] possibly more specifically through the binding of the $\alpha M\beta 2$/Mac-1 to sites in the γ chain,[106] specifically to the 383–395 sequence that is cryptic in fibrinogen but becomes exposed upon immobilization or specific enzymatic cleavage. It is worth mentioning that adhesion to fibrin, however, appears to be strongly dependant on flow conditions; for example, when fibrin is forming in the flow of a fibrinogen-containing medium, neutrophils can end up coating themselves in fibrin (but not in fibrinogen).[107]

However, cell adhesion (as much as migration or remodeling) is most often guided by biomolecules bound on fibrin. Indeed fibrin's biophysics has plenty of interaction partners, starting from fibrin own synthesis: during blood clotting a number of factors such as von Willebrand factor,[108] vitronectin,[109,110] coagulation factors VIII[111] and XIII[112,113] become incorporated in the fibrin network. In particular, cell adhesion can also be facilitated by fibrin-bound extracellular matrix (ECM) proteins such as vitronectin[109] and fibronectin,[114] which bind to a αC chain site exposed in fibrin but not in fibrinogen.[114]

In terms of other forms of cell-instructive functions, fibrin can also interact with several growth factors, chiefly those from the vascular endothelial growth factor (VEGF) and of the fibroblast growth factor (FGF) families.[115–117] The ability to sequester and act as a repository of several growth factors has proved beneficial for wound healing applications, as also discussed later in Section 4, "Fibrin as an artificial extracellular matrix." It should be noted that truncations or other modifications in the growth factor structure can obliterate binding and induce quantitative burst release, which means more complex strategies are required to immobilize those fragments in fibrin.[118]

3. Clinical Applications

3.1. A Medical Device

The Food and Drug Administration (FDA) approved the first fibrin sealant for clinical use in 1998, although Tisseel (from Baxter) was commercially available in Europe since 1972. The first use of fibrin from a surgical perspective (wound healing upon subcutaneous or intraperitoneal administration in guinea pigs and rabbits) goes considerably further back in time to 1909.[119]

The FDA has approved fibrin-based products for uses as a hemostat, sealant, and adhesive.[120] Hemostats are devices designed to accelerate the blood clotting process of blood; clearly, the presence of blood is required. Sealants intend to prevent the leakage of fluids by forming a barrier, which may adhere or be mechanically interlocked with tissues. Adhesives (or glues) are designed to adhere to structures and bring them together, thereby providing or restoring mechanical integrity. Although the application of sealants and adhesives may physically stop the flow of blood, they do not actively induce hemostasis. Fibrin is as the only clinically approved material that can serve all the three purposes, which makes it attractive for a variety of clinical scenarios. It is also important to emphasize that even limiting the analysis to the field of clinically approved tissue adhesives and sealants, fibrin is often the material of choice due to its safety profile upon application and during degradation; cyanoacrylate-based glues, for example, may be superior in adhesion, but often cause necrosis in the surrounding tissues (monomer toxicity) and may slowly release cyanoacetate and formaldehyde (from backbone hydrolysis).[121]

Due to its use in the applications above, the primary more of action of fibrin is typically classified as mechanical rather than biochemical/pharmacological; therefore, these products (see complete list of approved products in **Table 2**) are typically classified as medical devices, which makes the regulatory approval process less lengthy and expensive than, for example, pharmaceutical products.

3.2. Applications

We refer the reader to other works that more extensively review the application of fibrin sealants in clinical settings,[120,122,123] including their use as skin grafts and burn wound reconstruction,[124–126] cardiovascular surgery,[127] and abdominal surgery.[128–132] It is worth mentioning that the popularity of fibrin has significantly grown with the advances in laparoscopic surgery, where tissue closure must be based on minimally invasive techniques, since the restricted space and the limited visualization make classical suturing complex.[133]

Fibrin sealants are commonly applied by dripping or spraying. Dripping involves the use of a 2-barrel syringe that separately stores a fibrinogen and thrombin solution, combining them through a passive (laminar) mixer placed before the syringe needle. This method is particularly effective to localize fibrin formation and to fill volumetric spaces. Sprays are better at producing even layers of sealant; in 2012 the European Medicine Agency (EMA) issued specific advice following some reports of gas embolism after spray application of fibrin sealants, which are possibly caused by the use of higher than recommended pressure of air (but not of CO_2).

3.3. Issues and Solutions

1. *Possible interference with anticoagulant therapies*. Most fibrin glues are claimed to be effective also in heparinized patients, despite heparin's anticoagulant activity being based on thrombin inactivation.[134,135] This is possibly a consequence of the high thrombin concentrations used in fibrin glues (see note "a" to Table 2), orders of magnitude larger than in normal blood (typically below 10 IU mL^{-1}).
2. *Fast fibrin degradation*. Antifibrinolytic agents such as aprotinin may be introduced to slow it down. However, aprotinin is often of bovine origin and therefore susceptible to cause allergic reactions; indeed adverse events due to anaphylactic reaction to aprotinin have an incidence of 0.5%.[136]
3. *Human versus animal origin*. Concerns in the use of animal products (see point 2) have increased the use of human plasma-derived products; however, this carries the risk of transmission of viral and prion diseases. These risks are

Table 2. Fibrin-based products approved by FDA or by the European Medicine Agency.

Product, manufacturer	Approval	Use	[Fibrinogen]	Source	Notes[a)]
Tisseel/Tissuecol, Baxter Healthcare	FDA, 1998 EMA, 2008	Hemostat, sealant	67–106 mg mL^{-1}	Pooled hum. plasma	Contains aprotonin
Evicel, Orix Biopharm.	FDA, 2003 EMA, 2008	Hemostat	55–85 mg mL^{-1}	Pooled hum. plasma	A development of earlier versions Crosseal (US)/ Quixil
Artiss, Baxter Healthcare	FDA, 2008	Adhesive	67–106 mg mL^{-1}	Pooled hum. plasma	Contains aprotinin
RAPLIXA, The Medicines Co	FDA, 2015	Sealant	79 mg mL^{-1}	Pooled hum. plasma	To be used with a gelatin sponge
VistaSeal/VeraSeal, Instituto Grifols/Ethicon	FDA, 2017 (EMA, retr. 2015)	Hemostat	80 mg mL^{-1}	Pooled hum. plasma	Ethicon provides the application device
Beriplast P, CSL Behring	FDA, 2017	Hemostat, adhesive, sealant	90 mg mL^{-1}	Pooled hum. plasma	Contains bovine aprotonin and Factor XIII
Fibrinogen-only products					
RiaSTAP, CSL Behring	FDA, 2009 EMA, 2010	Hemostat[b)]	18–20 mg mL^{-1}	Pooled hum. plasma	
FIBRYNA, Octapharma Pharm. Prod.	FDA, 2017	Hemostat[b)]	20 mg mL^{-1}	Pooled hum. plasma	
Autologous fibrin					
Cryoseal, ThermoGenesis	FDA, 2007	Hemostat, sealant	N/A	Patient's own blood	Device to produce autol. cryoprec. fibrinogen and separate thrombin from patient's samples
Vivostat, Vivostat A/S	? [c)]	Hemostat, sealant	N/A	Patient's own blood	Device to produce autol. platelet-rich fibrin from patient's samples without cryoprecipitation
Fibrin patches					
TACHOSIL, Takeda Pharma	FDA, 2015 EMA, 2004	Sealant patch	3.6–7.4 mg cm^{-2}	Pooled hum. plasma, equine collagen	Collagen sponge with fibrinogen and thrombin
EVARREST, Omrix Biopharm.	FDA, 2016 EMA, 2013	Sealant patch	8.6 mg cm^{-2}		Oxidized regenerated cellulose on polyglactin 910 patch with fibrinogen and thrombin

[a)]When thrombin is present in fibrin products, its concentration is typically 400–600 IU mL^{-1}, except for Evicel (around 1000 IU mL^{-1}) and Artiss (around 5 IU mL^{-1}—delayed gelation to improve adhesion). The thrombin concentrations for fibrin patches are expressed per surface unit, and markedly varies between products. Tachosil—around 2 IU cm^{-2}, Evarrest—around 37 IU cm^{-2}; [b)]Concentrated fibrinogen used for treating fibrinogen deficiency; [c)]The authors have found no information about FDA or EMA approval, although clinical trials have been concluded.

minimized through the certified donor selection programs and viral inactivation procedures during manufacturing, with no HIV or hepatitis transmission being reported in 20 years of clinical use worldwide.[137] A further step is the use of autologous fibrin sealants (from patient's own blood), which were recently reviewed by Padilla et al.[138]; the possibility to use an autologous source is a key advantage of fibrin over other potentially injectable materials such as collagen or gelatin, which typically are only available from animal sources.

4. *Fibrinogen concentration*. Fibrinogen is typically employed at a concentration at least an order of magnitude above what is typically found in blood. The stiffness of a blood clot mostly depends on its cellular content, with fibrin acting as a glue. In the absence of cells, a sufficient mechanical strength (e.g. moduli of a few kPa) can only be obtained with fibrinogen concentrations exceeding 20 mg ml^{-1}. However, at such high concentrations, the pores of the fibrin networks have such a small size to hinder the infiltration of cells and the diffusion of macromolecules, including proteolytic enzymes. Furthermore, even with these artificially high concentration, fibrin sealants can be considerably softer than the tissues they are glued on, and the chances of an adhesive failure increase with fibrin degration.[120,123] An important drawback of fibrin softness, is that it makes it susceptible to cell-mediated contraction. However, initial clot contractility is essential for hemostasis and wound closure, but it may continue leading to chronic inflammatory state and ultimately to fibrosis, scarring and tissue adhesions.[139]
5. *Injectable (in situ formed) versus pre-formed fibrin products*. A major advantage of fibrin is the possibility to apply a solution (injected through syringes, sprayed, dripped through a catheter) and form a solid-like material at the desired anatomical

location. This allows for a minimally invasive administration and for a conformal nature of the sealant. However, a few non-injectable fibrin products exist too (last portion of Table 2); these patches combine an implantable material in the form of a membrane or a foam (providing superior strength) with a fibrinogen-containing solution—typically human pooled plasma clotting in situ (allowing for tissue adhesion). Therefore, these patches can be regarded as non-injectable formulations with an injectable add-on, that is, fibrin. The loss of injectability is counterbalanced by 1) the better mechanical properties and 2) the possibility to store the materials for a prolonged time at room temperature, temperature, which means that they do not need a cold chain because plasma is typically sourced at the hospital for the intervention. As a comparison, only lyophilized fibrinogen formulations can be kept for long at room temperature (e.g., Tisseel Lyo for up to 2 years), whereas −20 °C storage is always necessary for solutions.

6. *Drug release*. As an in situ forming gel, fibrin offers an attractive opportunity for the local delivery drugs,[140] for example, lidocaine,[141,142] sisomicin,[143] doxorubicin,[144] and even growth factors.[145] However, in the absence of specific interactions with the matrix, the release kinetics of these compounds is often too fast to be really useful; therefore, affinity binding or covalent functionalization are essentially the only means to achieve a sustained release kinetics, see Section 4.1 "Fibrin as an in situ forming delivery vehicle."

4. Fibrin as an Artificial Extracellular Matrix

Fibrin is possibly the best natural example of provisional matrix, and it is therefore hardly surprising that it has been widely exploited as an in situ forming, degradable ECM, for example, in skeletal muscle,[146–148] bone,[149] skin,[126,150] nerve,[151–153] and cardiac tissue engineering.[154] In a comparative study of five natural polymers for skeletal muscle tissue engineering, fibrin was found to be the one with the highest myogenic potential.[147]

A short list of additional favorable points should mention a) the facility its properties can be modulated via the straightforward alterations in the polymerisation conditions, as comprehensively reviewed by Barker.[155] b) its contractility. Often regarded as negative, in several applications it can be highly beneficial, for example, for wound closure and the production of aligned tissue constructs where cell-mediated contraction between two fixed anchors can lead to the development of aligned tissue models such as skeletal muscle,[148,156] tendon and ligament.[157,158] c) the reduction of wound contamination, since fibrin films inhibit the invasion of bacteria.[159]

4.1. Fibrin as an In Situ Forming Delivery Vehicle

4.1.1. Growth Factors and Low MW Drugs

Fibrin can bind, retain, present, and possibly release biomolecules that have profound effects on cell behavior, chiefly growth factors such as VEGF,[50,116,160] bFGF,[117] platelet derived growth factor (PDGF),[160] platelet factor 4 (PF4),[50,161] and a few others. At least for some of them this binding is mediated by a) a heparin-binding domain found in the β15–66 region of fibrinogen (**Figure 3**A),[160] b) the presence of a heparin-binding domain in the growth factor: for example, when the strongly heparin-binding 123–144 sequence of placenta growth factor-2 (PIGF-2) is fused with several other growth factors, the latter end up strongly binding fibrin[162] and being released upon fibrinolysis. This is compatible with a "sandwich" model (Figure 3A), where an anionic compound such as heparin acts a glue between two cationic, heparin-binding domains (one on fibrin, one on the growth factor); this mode of action is supported by the retarding effect of heparin on the release of bFGF from fibrin.[163]

In a less biofunctional fashion, fibrin has been used as an injectable matrix hosting, retaining and—upon degradation—delivering drug loaded-microparticles throughout a long period of time, up to a few weeks for bupivacaine-loaded poly(lactide-*co*-glycolic acid) (PLGA) particles.[164]

Fibrin hydrogels have been applied in wound dressings to release antimicrobial agents, that can range from established antimicrobial drugs,[168–170] to bacteriophages,[166] and even the very cells still present in leucocyte- and platelet-rich fibrin.[167] The interactions of fibrin(ogen) itself with bacteria, however, have not been completely clarified, and there are evidences pointing to opposite directions:for example, on one hand *Staphylococcus aureus* can activate prothrombin via coagulases to form fibrin "shields" that help its evasion from phagocytic cells[171]; on the other hand the adhesion and subsequent clumping of *S. aureus* to fibrin(ogen), mediated by clumping factor A, has been shown to facilitate the clearance of infection after peritonitis.[172]

4.1.2. Cells

Since fibrin can be easily formed under physiological conditions and is characterized by both biocompatibility and cell adhesion, it has been often used for the localized delivery of a variety of precursor/stem cells, for example, in esophageal reconstruction,[173] peripheral nerves,[174] and above all in skin; there, cells have been delivered in (pre-vascularized) fibrin matrices,[175] within fibrin microbeads,[176] or sprayed in gelling fibrin (although will little advantages over their spraying without fibrin);[177] the most widespread use of fibrin in skin, however is still as a glue for (autologous) skin grafts.[125,126,178]

Finally, it is worth mentioning a recent approach by the group of Perriman, who instead of dispersing cells within a gelling fibrinogen solution, have nucleated fibrin around them: using a complex between a heavily cationized thrombin and a PEGylated surfactant that accumulates on cell surfaces, fibrin was produced directly on the surface of mesenchymal stem cells. With this method, 3D constructs can be produced where cells are individually encapsulated, and then driven toward various differentiation paths through an appropriate choice of the culture media.[179]

4.2. Modified or Hybrid Fibrin Materials

Despite a number of favorable properties, fibrin mechanical properties and degradation are often considered to be

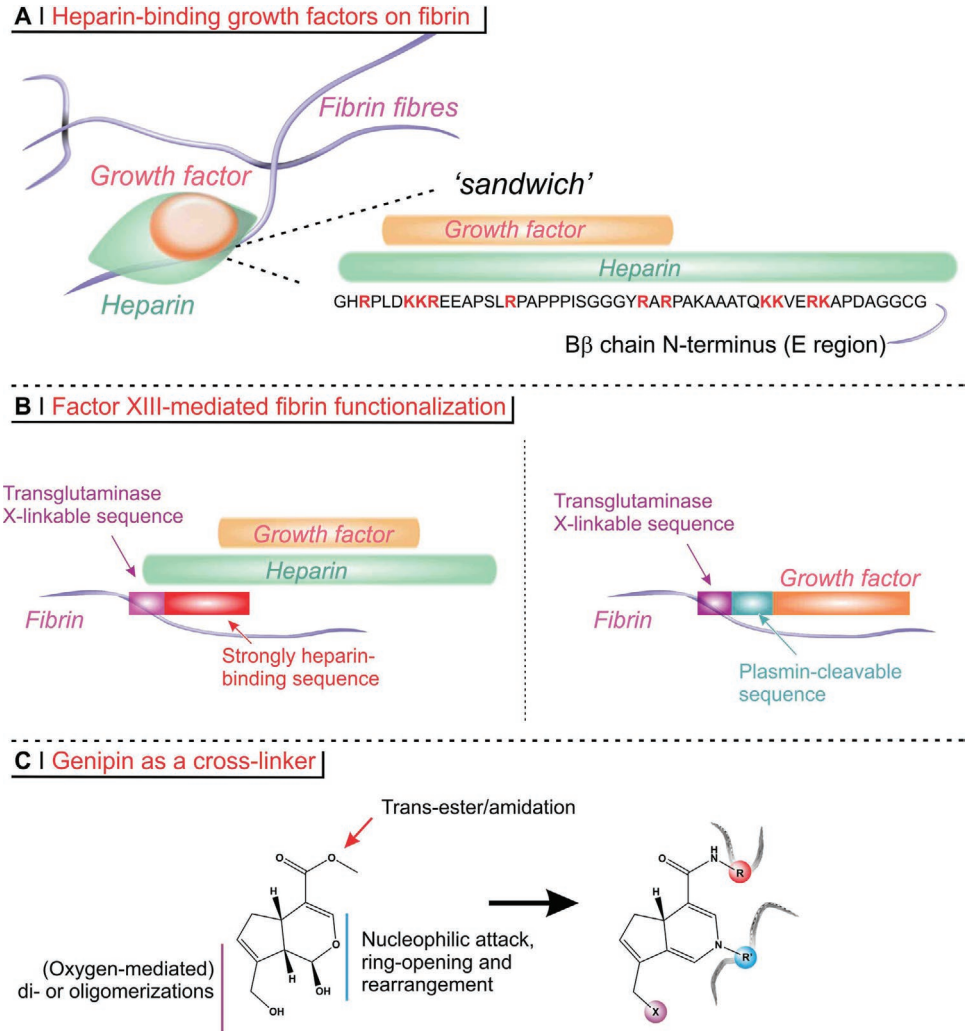

Figure 3. A) Likely mode of action for growth factor (GF) loading on fibrin gels; a sequence with a high content of cationic residues (in red) is present at the N-terminus of Bβ chains after the thrombin-mediated cleavage of fibrinopeptides B; this sequence is considered to be a heparin-binding site, and possibly forms a sandwich-like structure where heparin bridges this heparin-binding sequence of fibrin with those of growth factors such as VEGF and bFGF. B) Factor XIII has been used to covalently connect structures to fibrin, possibly but not necessarily to its αC regions. "Sandwich" structures as those described in (A) can be obtained by introducing peptides with a strong heparin-binding activity (left). Alternatively, fusion proteins can be introduced, which at each end contain the active molecule and the Factor XIII substrate, with a cleavable space that allows the release of the active portion during fibrin degradation (right). C) Structure of genipin (left) and sites of its possible cross-linking reactions[165] (right).

improvable, in particular too soft and too rapidly degraded. These points are often tackled by using additional polymeric components and/or cross-linking agents, which in some cases can also allow to introduce additional functionalities.

4.2.1. Functionalization or Cross-Linking via Factor XIII

Factor XIII stabilizes fibrin via covalent cross-linking (see Section 2.1 "Fibrinogen and its Conversion into Fibrin"); supplementation of Factor XIII is well known to yield matrices that are stronger[33] and less prone to degradation,[180] with higher mass-to-length ratio fibers.[78] The Factor XIII dose-dependent increase in modulus can be negated by using Factor XIII inhibitors.[31] It is here worth to mention that Factor XIII has also been used to cross-link synthetic polymers (e.g., multifunctional PEGs[181]) in a fibrin-mimetic fashion.

The use of N-terminal transglutaminase domains is a strategy pioneered by the Hubbell's group, which has allowed

1. To functionalize fibrin fibers with strongly heparin-binding peptides (e.g., inspired by antithrombin III structure[182] or alpha(2)-plasmin inhibitor, dLNQEQVSPLRGD-NH_2[183]) and then decorate them with heparin and an heparin-binding growth factor (bFGF,[182] nerve growth factor[184]) in a sort of "sandwich" structure (Figure 3B). As previously discussed, bFGF can already be directly loaded on fibrin, most probably in a heparin "sandwich" as depicted in Figure 3A, but the use of strong heparin-binding sequences in this strategy likely increases the growth factor retention.

2. The direct attachment of growth factors such as VEGF,[185] insulin-like growth factor-1 (IGF-1),[186] BMP-2,[187,188] BMP-2/MP-7 heterodimers,[187] by gifting them with a Factor XIII substrate (e.g., an N-terminal NQEQVSPL sequence[185]), typically accompanied by an enzymatically cleavable sequence for a plasmin- or MMP-induced release of the growth factor. Also other peptidic substrates have been added to fibrin with the same technique, for example, laminin and N-cadherin sequences[189] or $\alpha_v\beta_3$ integrin binders such as TG-L1Ig6,[190] in order to enhance neuron adhesion and neurite extension.

4.2.2. Cross-Linking with Low MW Compounds

Genipin is widely employed to cross-link virtually any kind of amine-containing macromolecules, including fibrinogen or fibrin. Genipin derives from a naturally occurring compound (geniposide, a glycoside present in various fruits) via β-glucosidase treatment, and can react with amines at two sites of its molecular structure and then further cross-link a material through oxidative oligomerization (Figure 3C). Due to the unselective nature of these reactions, genipin crosslinking can increase both fibrin modulus and its resistance to degradation,[191–193] but can also allow its covalent mixing with other materials, for example, silk,[194] alginate,[195] and decellularized ECM particles,[196] thereby minimizing phase separation phenomena. Poly(methyl methacrylate) (PMMA) beads have also been incorporated in fibrin prior to the addition of genipin; after cross-linking, and dissolution of the beads in acetone, this allows to introduce a controlled microporosity in fibrin gels.[197]

4.2.3. PEGylated Fibrinogen-Based Materials

Technically, these materials are not based on fibrin, but rather are hybrid constructs containing intact fibrinogen. Poly(ethylene glycol) (PEG) chains are either employed as bifunctional reagents that bridge fibrinogen macromolecules, and therefore perform PEGylation and curing in one operation (PEG-bridged fibrinogen gels; **Figure 4**A), or are initially introduced as pendant chains in what actually is a macromonomeric structure, which is then cross-linked by the means of the reactivity of PEG terminal groups (PEG-fibrinogen macromonomer gels; Figure 4B).

Gels obtained through the first approach (e.g., using PEG bis active esters reacting with fibrinogen amines) have been used as scaffolds for cell delivery,[198–201] applying them, for example, as wound dressings that can reduce both contraction[202–204] and inflammation,[204] with positive effects also on microbial contamination.[205–207] Since PEGylated materials have reduced interactions with most biomolecules, including proteolytic enzymes on fibrin or fibrinogen, it is not surprising that these materials are gifted by high stability against degradation.[198] However, it is noticeable that when a relatively low amount of bifunctional PEG is added (1:10 in weight), the PEGylated fibrinogen can still undergo thrombin-mediated conversion to fibrin.[208]

In the second approach, pioneered by the group of Seliktar, an excess PEG diacrylate is typically employed; the acrylic groups partly react with fibrinogen through Michael-type addition therefore generating PEG chains that at one hand are tethered to fibrinogen and at the other bear an intact acrylate. These constructs are effectively curable PEG-fibrinogen conjugates, and are then typically cross-linked via photopolymerization, thereby ending up being integrated within a network composed of PEG and polyacrylate chains.[209,210] In this case, the increased stability of fibrinogen against degradation is due to both the direct effect of PEGylation and to the presence of a synthetic polymer network.[209] It is noteworthy that, despite PEG/acrylate networks being essentially non-adhesive and non-degradable, the integration of fibrinogen allows a) cell adhesion and significant degradability,[210] b) the means to tune the latter in order to control the kinetics of the release of (osteogenic) fibrinogen fragments.[211] Due to the benign character of photopolymerization, living cells can be easily encapsulated and imaged in these materials,[212] that can also be fashioned in the form of microparticles[213] or spheroid/microspheres.[214] These PEGylated fibrinogen gels have been employed in a variety of in vitro models, for example, including pluripotency maintenance (human mesenchymal stem cells)[215] or neural growth neural outgrowth,[216] and have been applied in vivo for BMP-2 stimulated bone repair,[217] for the generation of skeletal muscles from encapsulated mesangioblasts,[218] for the regeneration of sciatic nerve,[219] or for myocardial regeneration (no cell encapsulated) after infarction.[220]

4.2.4. Fibrin Hybrid Materials

1. *The problem of phase separation*. The general aim to obtain a wider spectrum of degradation kinetics and (nonlinear) mechanical properties and of direct cell interactions with a finer control has led to the development of a large number of hybrid materials where matrices are present and often interpenetrating.[221] In this area, an issue is often overlooked: producing hybrid materials by "simply" mixing components is often more complex than most imagine. This goes back to the entropy of mixing for large polymers not only in the solid state but also for their concentrated and semi-diluted solutions: in the absence of specific interactions (affinity), phase separation is the rule and "real" mixing at a (supra)molecular level the exception. Although these phenomena often go unnoticed, there are some commendable reports that make it clear that mixtures of fibrin and other more or less well-associating polymers (e.g., chitosan[222]) are clearly phase separated. Importantly, phase separation most commonly occurs during the conversion of fibrinogen to fibrin. The criticality of this point is that phase separation and above all its kinetics are rather difficult to precisely control,[223] which on its turn may lead to poor control over structural and mechanical properties of the materials.

   It must be noted that in some cases phase separated fibrin-containing hybrids have been produced intentionally and in a controlled fashion. This applies, for example, to fibrin-collagen composites prepared using multilayered constructs,[186] or microfluidic-produced fibrin microbeads[233] dispersed in collagen.[234] This phase-separated structure allows to obtain a bulking agent (e.g., for bladder) that takes advantages of

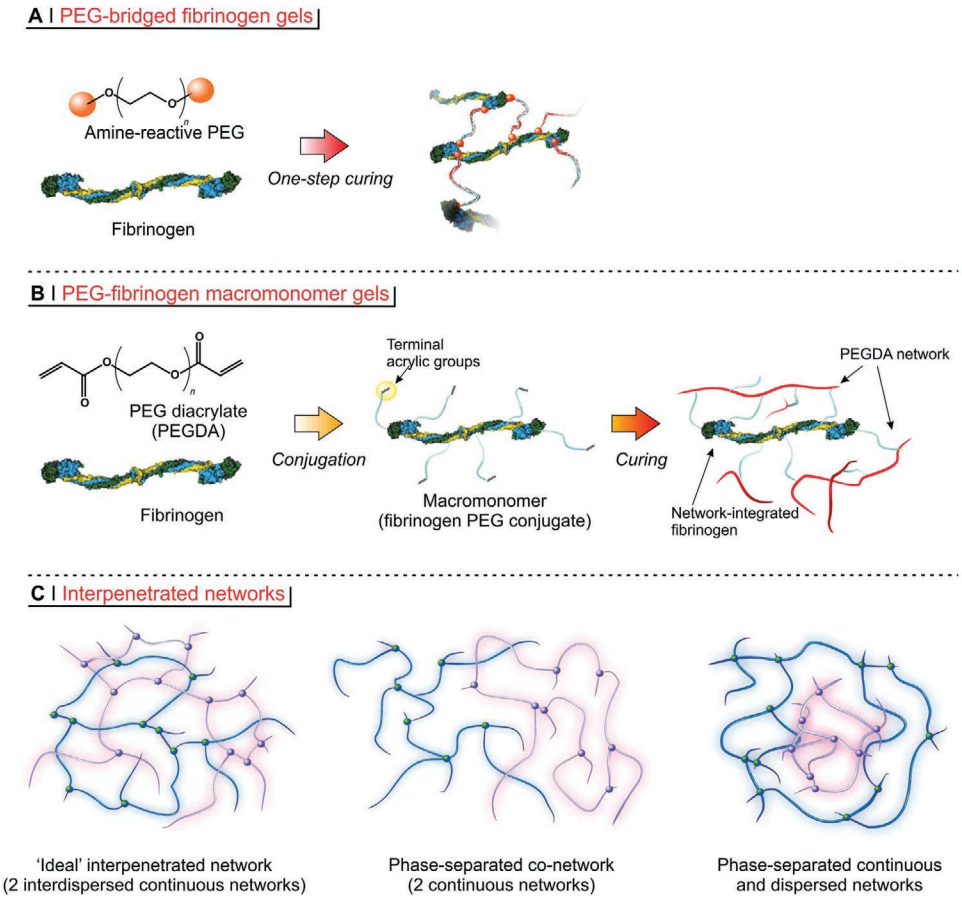

Figure 4. A) A PEG-bridged fibrinogen network is compositionally homogeneous and fibrinogen molecules effectively act as a multifunctional, macro cross-linkers, which are connected by PEG bridges. B) A PEG-fibrinogen macromonomer can be obtained using an excess of PEGDA in comparison to fibrinogen lysine residues, and thereby producing a PEGylated fibrinogen with plenty of pendant acrylic groups. When the latter are cured (in the presence of unreacted PEGDA), one obtains a network with some composition heterogeneities, which are represented by fibrinogen integrated within a PEGDA network. C) In an ideal case, interpenetrated networks are inter-dispersed down to the individual components (*left*), but significant phase separation can occur for entropic reasons during cross-linking/fibrillation/polymerization processes, leading to the formation of (partially) phase-separated continuous networks (= both are above a percolation threshold, *middle*), or of a dispersed and a continuous phase (*right*). In the last case, the overall material properties are typically more influenced by the continuous than the dispersed phase.

collagen mechanical properties and of the facility of fibrin molecular engineering (in these cases fibrin was functionalized with a fusion protein containing IGF-1, an MMP-cleavable bridge and a Factor XIII substrate).

2. *Interpenetrating networks.* "Ideal" IPNs should be co-continuous networks of, for example, fibrin and another component[224]; their composition should be roughly homogeneous at any level of scale, that is, taking molecule A and molecule B at a given composition, the same A/B ratio should be found at a macroscopic, microscopic or nanoscopic scale (Figure 4C, left). In real cases, IPNs may have a degree of phase separation (Figure 4C, middle), and this may extend to the point of one component being segregated in isolated domains (Figure 4C, right).[225] Fibrin-based IPNs typically show higher moduli and an improved behavior with cells (above all less or slower contraction), and among the second components one should mention hyaluronic acid, which was cross-linked via disulfide formation (thiol + 2-pyridyldisulide) during fibrin formation;[226,227] alginate, which gels thanks to the high calcium concentration of fibrinogen solutions[228]; chitosan, which was cross-linked by reacting in situ with a bis(formyl)PEG;[229] PEG[230,231] and poly(vinyl alcohol) (PVA),[232] which are cured via photopolymerization of respectively terminal or side-chain methacrylates.
3. *3D-printed structures.* 3D-printed, fibrin-based constructs have been employed for the production of a variety of tissues or tissue-like structures, including micro vasculature,[235] nerves,[236,237] urethra,[238] and bone.[239] In order to ensure printability and to produce self-supporting structures, these bioinks are rarely composed only of fibrinogen/thrombin mixtures, but include other water-soluble polymers such as alginate, hyaluronic acid or gelatin. These potentially cell-laden solutions are often printed within or around more rigid scaffolds, for example, made of degradable polyesters,[238,240] the latter being ultimately responsible of the mechanical properties in the final constructs. It is important to realize that also in this case controlling the phase behavior of the components is a critical step to ensure reproducibility.

4.3. Fibrin Engineered through Affinity Interactions

An elegant way to get around phase separation, and therefore to allow the precise introduction of new functions in a fibrin gel, is to provide the additional components with specific affinity towards fibrin. This concept was pioneered in 1980 by Laudano and Doolittle, who altered structure and properties of fibrin clots through affinity interactions,[241] for example, showing that Gly-Pro-Arg (GPR) sequences (A knob mimics) would disrupt the gelation processes whereas ones starting with Gly-His-Arg-Pro (GHRP, B knob mimics) did not. These peptide/fibrin knob–hole interactions have been quantified by Kokonova et al.[242] showing that a:A interactions are stronger than b:B at neutrality, but weaker at pH 5. Of note, although GPRP and GHRP are the physiologically relevant human knob A and B mimics, respectively, the homologous chicken-fibrin AHRP for knob B has more specific interactions with the b hole.[243]

4.3.1. PEGylated Constructs

In this area, most literature reports have utilized conjugates of fibrinopeptide with PEG, because its "stealth" nature of PEG can remove effects from additional interactions from those solely based on knob:hole coupling. When monofunctional PEG was employed, it is also possible to depurate binding data from avidity contributions.

PEGylated GPRP (A knob mimic) slows down in a dose-dependent fashion fibrin gelation.[77,244] This and the very early evidence by Doolittle suggested the possibility to employ these conjugates as anticoagulants; using three PEGylated A knob mimics, GPRPAAC, GPRPFPAC, and GPRPPERC (the last two more active), it has been showed that 5 kDa PEG to perform better than shorter PEGs or unconjugated peptides (possibly suffering of more interference) and larger PEGs.[245] The lesser effect of larger PEGs was initially ascribed to their steric hindrance,[245] but in a later study the same group (Barker) showed that the larger PEGs had a significant effect on lateral aggregation, reducing fiber diameter[246]; this may lead to a higher volume density of (thinner) fibers, which may be the explanation of the scarce anticoagulant effects of these large PEG conjugates. Of note, PEGylated GPRP has shown to have a small influence on Factor XIII, but increasingly with PEG molecular weight[244]: probably, smaller fibers have a less pronounced effect of transglutaminase cross-linking.

There is plenty of evidence that GPRP peptide conjugates (A:a interactions) lower fibrin modulus and produce (SEM evidence) truncated/capped fibers.[77,246] Our group has seen the PEGylated GHRP had a similar, but lesser effect,[77] whereas PEGylated AHRP (both peptides in principle being B knob mimetic) have been reported to increase the modulus[246]; this apparent diversity is probably to ascribe to the different specificity of the two peptides, but it must be said that the effects of b:B interactions of these PEGylated peptides are often complex to control with a variety of parameters involved.[247]

The dose-dependent effect of both 2 kDa PEG-GPRP and -GHRP on fibrin stiffness is predominantly down to a lower fiber bending modulus as a consequence of defects introduced by the peptides (and possibly a lower lateral aggregation), since the overall character of the network (Poisson's ratio, relaxation time, overall morphology) is unchanged. In parallel to the decrease in modulus, we observed an increase in fibroblast mobility in these 3D matrices, which fits very well a model of non-proteolytic, adhesion-mediated motility: the mesenchymal cells move easier through networks where fibers can be more easily bent.[77]

A more extensive summary of the effects of fibrinopeptides on fibrin properties is presented in **Table 3**.

4.3.2. New Fibrin-Targeting Groups

Besides the classical fibrin own N-terminal groups, a number of alternative epitopes can specifically recognize fibrin or fibrinogen, as reviewed by Barker.[248] They include peptides targeting fibronectin-, plasminogen- and antiplasminogen binding domains, or fibrinogen fragments from freeze fracturing and evolutionary approaches which use phage biopanning.[248]

In particular, phage-displayed biopanning is a useful methodology for the identification of targeting ligands such as H6,[249,250] SP2,[251] CREKA,[252] and CLT-1[253] for fibrin. The H6 sdFvs recognition motif for fibrin was recently utilized to produce platelet-like microparticles that act as strong artificial hemostatic agents to enhance clot stability for the treatment of severe haemorrhage.[254] These PEG-based platelet-mimicking microgels reinforce the fibrin network to cause an ≈fourfold increase in elastic modulus, a ≈threefold increase in network permeability, and a resistance to plasmin degradation over 24 h.[254] The group of Pun developed a similar concept by using a disulfide-containing cyclic peptide (Y(DGl) C(HPr)YGLCYIQGK) peptide identified by phage display for its selective binding to fibrin but not to fibrinogen identified[260]; the peptide was introduced on 2-hydroxyethylmethacrylate (HEMA)-based polymers, which were used for strengthening fibrin clots.[261–263] CREKA (Cys-Arg-Glu-Lys-Ala) peptides bind fibrin-fibronectin binding peptides and have been employed for tumour targeting, by taking advantage of the elevated and persistent fibrinogen extravasation (and therefore fibrin accumulation) from the fenestrated microvasculature within tumors.[252,264,265]

For tissue engineering purposes, an attractive approach of fibrin modulation is to introduce ECM-mimicking binding motifs to influence the cellular binding and subsequent response. For instance, laminin-111 peptides (peptide A99 and YIGSR) were conjugated onto a thiol-reactive fibrinogen were able to form hydrogels that enhanced the attachment and differentiation of salivary[266] and parotid gland[267] clusters. The peptide conjugation significantly decreased the shear modulus of the resulting gel, which facilitated the formation of salivary glands tissue in vivo although they were unable to recapitulate the function of these glands.[267]

5. Conclusions

Fibrin is more than a promising biomaterial. On one hand, it is "the" provisional matrix *par excellence*, and on the other hand it is a medical device approved by regulatory agencies and with

Table 3. Use of fibrinopeptide and their conjugates to modulate fibrin network properties.

Sequence	Conjugation	Effects	Ref.
GPRPGCG[a] GHRPGCG[b] GSPSGCG[c]	Monovalent 2 kDa PEG. Michael-type addition of C on vinyl sulfone	GPRP ↑: fiber diameter ↑, shear modulus ↓, cell migration ↑ Similar but less pronounced with GHRP	[77]
GPRPAAC[a] GSPSAAC[c]	Bi-, quadrivalent 2–20 kDa PEG. Michael-type addition of C on maleimide	2 kDa PEG: GPRP ↑: fiber diameter ↑ >5 kDa PEG: GPRP ↑: fiber diameter ↓	[244]
AHRPYAAC[b] GPSPFPAC[c]	Monovalent 5 kDa PEG. Michael-type addition of C on maleimide	AHRPYAAC ↑: fiber diameter ↑, network porosity ↑, shear modulus ↓	[247]
GPRPAAC[a], GPRPFPAC[a], GPRPPERC[a]	Monovalent 2–30 kDa PEG. Michael-type addition of C on maleimide	GPRPAAC—most potent effect 5 kDa PEG conjugated peptides—largest effect at 1:100 molar ratio Peptide ↑: fiber diameter ↓, clotting time ↑	[245]
GPRPGGGGC[a] GHRPYGGGC[b] AHRP[b]	Bovine serum albumin (BSA). Disulfide formation with BSA free thiols (C in an amidated form). AHRP not conjugated to BSA	GPRPGGGGC, fiber diameter ↑; GHRPYGGGC, fiber diameter ↑ (slightly more than GPRPGGGGC)Free albumin ↑– fiber diameter ↓	[255]
GHRP[a], GHRPL[b], GHRPY[a]	None	Peptide ↑: fiber diameter ↓, plasmin degradation ↓ and delay in fibrin tPA activation. Potency: GHRPY ≫ GHRPL > GHRP	[256]
GPRVVAAC[a], GPRVVERC[a], GPRPAAC[a], GPRPPERC[a], GPRPFPAC[a], GPSPAAC[c]	None Cysteines present for possible conjugation, but not used	GPRPFPAC highest affinity (more than the biomimetic GPRV-peptides)	[257]
GPRPFPAC[a], AHRPYAAC[b], GPSPFPAC[c]	Core-shell microgel, shell of poly(***N***-isopropyl methacrylamide-***co***-acrylic acid). Peptides coupling through amidation, cysteines not used	Shell-functional microgels increased the flow through fibrin gel-obstructed channels. Potency: GPRP > AHRP > GPRP microgels	[258]
GPRP[a] and RGD	Micro-plasminogen (μPlg)	RGD-μPlg best inhibitor for ADP-induced platelet aggregation (33-fold relative to μPlg). GPRP-μPlg strongest effect on gelation time (ninefold relative to μPlg)	[259]

[a]Knob A mimic; [b]Knob B mimic; [c]Non-binding control.

a widespread clinical use. Clearly, details matter. For example, blood coagulation is not based on fibrin alone, but rather fibrin is the functional but quantitatively minor element of a composite material. Fibrin glues (hemostats, sealants) are different, in that they are fibrin-only materials, but produced using fibrinogen concentrations far higher than its physiological levels. In tissue engineering/regenerative medicine, fibrin is one of the most commonly employed matrices, it allows to recapitulate in vitro a number of cues of "normal" ECMs, and has a history of use as injectable, in situ forming matrix for in vivo experiments. Yet, in this field such a versatile materials has failed to become a 'gold standard, probably because of its shortcomings in terms of mechanical properties (weak), cell-mediated contraction, rapid degradation, and phase separation when in mixture with other biomolecules. However, by integrating strategies of molecular engineering, it is possible to address most if not all these points. For example, components with specific affinity interactions may at the same time avoid phase separation and provide additional functions. Specifically, we want to point that the development of fibrin-specific binders is an important breakthrough, bearer of major results in the years to come.

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Conflict of Interest

The authors declare no conflict of interest.

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