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Modulation of fibrin matrix properties via knob:hole affinity interactions using peptide-PEG conjugates

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Abstract

Fibrin is a widely used biological scaffold in tissue engineering and regenerative medicine. While the polymerization dynamics from its soluble precursor fibrinogen has been studied for decades, few attempts have been made to modulate fibrin network structure through the addition of external agents that actively engage this process. We propose the use of polyethylene glycol (PEG)-based linkers that interact with fibrinogen via knob:hole affinity interactions as a means of controlling thrombin-mediated fibrin polymerization dynamics and resulting network structure. Using bivalent and tetravalent knob-PEG conjugates with sizes ranging from 2 to 20 kDa, we demonstrate that the clotting characteristics of fibrinogen solutions can be altered in a dose-dependent manner, with conjugate size playing a major role in altering fibrin network structure. Interestingly, factor XIIIa-catalyzed fibrin(ogen) crosslinking and plasmin-mediated degradation were not significantly impacted. This work demonstrates the feasibility of modulating fibrin network structure through the addition of knob-PEG conjugates that perturb the polymerization process through non-covalent knob:hole interactions.

Keywords

Fibrin; Fibrinogen; Polyethylene oxide; Hydrogel

1. Introduction

Many strategies in regenerative medicine and tissue engineering focus on the use of temporary scaffolds or provisional matrices to harness the natural healing resources of the body for tissue repair and regeneration. Whereas synthetic polymer systems are valued for their ease of synthesis, fabrication and modification [1], natural scaffolding materials are valued for their unmatched integration with the host biology [2]. For example, fibrin is the native provisional matrix generated in response to injury and has been evolutionarily optimized for stimulating and supporting cellular infiltration as part of the wound healing response. These facts plus its ease of purification have lead to the fibrinogen/fibrin system's widespread use in clinical applications [3], most notably as a bioactive tissue sealant (e.g. Tisseel®, Evicel™) [4].

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Fibrinogen, the 340 kDa inactive precursor of fibrin monomer, circulates in the blood at approximately 3 mg/mL and structurally comprises a symmetrical pair of three polypeptide chains - A α , B β and γ . Upon activation to fibrin monomer, the serine protease thrombin cleaves four N-terminal fibrinopeptides from the fibrinogen A α and B β chains, exposing knob peptide sequences, i.e. knob 'A' and knob 'B'. These fibrin knobs bind to complementary polymerization holes (hole 'a' and hole 'b') located at the two distal C-terminal regions of adjacent fibrinogen or fibrin molecules via very specific but non-covalent interactions. Sequential knob:hole interactions result in the formation of protofibrils with a half-staggered orientation that make up the fibers of the fibrin matrix (reviewed in [5]). The transglutaminase factor XIIIa simultaneously catalyzes the formation of isopeptide crosslinks between adjacent substrate sites on the C-terminal regions of both the α (α C) and γ chains, stabilizing the final network (Fig. 1A).

Fibrin network structure is strongly dependent upon fibrinogen, thrombin and calcium concentrations [6]. In particular, the knob:hole interactions initiated by the removal of fibrinopeptides are instrumental in determining key network parameters such as fiber diameter and branching density, both of which may be rapidly inferred from clot turbidity measurements [7]. The addition of knob peptide mimics such as GPRP (glycine-proline-arginine-proline; knob A mimic) and GHRP (glycine-histidine-arginine-proline; knob B mimic) perturbs clot turbidity and fibrin matrix formation in slightly different ways. GPRP-derived mimics inhibit fibril assembly through competitive binding, disrupting knob:hole interactions and increasing fibrinogen clotting time [8, 9]. On the other hand, GHRP-derived mimics enhance clot turbidity and delay plasmin-induced clot degradation presumably due to activation of α C domains enabling an alternative α C- α C based polymerization mechanism [10, 11].

Crystal structures of fibrin bound with GPRP-amide indicate that the distance spanning the distal holes (holes a) near the end of two abutting fibrinogen molecules is just 32 Å [12]. A separate group also found that a bivalent GPRP-conjugated 900 Da PEG linker promotes the end-to-end crosslinking of fibrinogen γ chains by factor XIIIa, suggesting that this length of PEG spans the distance between two adjacent holes a within a fibrin network [13]. Previously, our group has shown that GPRP binds specifically to fibrin(ogen) even when presented in the context of a non-binding 20 kDa protein, implying that this short peptide sequence is sufficient for mediating knob:hole interactions with both fibrin and fibrinogen [14]. Here, we propose the conjugation of sulfhydryl-terminated GPRPAAC peptides to the maleimide-terminated ends of various PEG structures, creating multivalent GPRP_n-PEG linkers capable of perturbing fibrin matrix structure through knob:hole interactions. This unique non-covalent means of altering matrix structure should preserve the physiological complexity of the original protein while allowing the independent control of matrix mechanical properties by altering conjugate size and valency. In particular, we focused on 2-arm and 4-arm PEG architectures with a minimum size of 1000 Da to permit the formation of hybrid networks of PEG and fibrinogen/fibrin (Fig. 1B). We hypothesize that these distance-spanning GPRP_n-PEG conjugates will interact with soluble fibrinogen and fibrin monomer and impose structural changes to the fibrin polymer network structure generated in the presence of thrombin and/or factor XIIIa.

2. Materials and Methods

2.1. Sulfhydryl-maleimide conjugation

Sulfhydryl-containing cysteine-terminated peptides (GPRPAAC and the control peptide GPSPAAC) were custom-ordered from Genscript (Piscataway, NJ) in pre-aliquoted and lyophilized form. Maleimide-activated components used were namely, 2, 3.5, 5, 7.5 kDa maleimide-PEG-maleimide, and 2, 10, 20 kDa 4-arm PEG maleimide (sourced from

JenKem Technology, Allen, TX; 2 kDa 4-arm PEG from Creative PEGWorks, Winston Salem, NC).

The conjugation buffer comprised 100 mM phosphate buffer pH 7.2, 150 mM sodium chloride, 10 mM EDTA. Desired quantities of sulfhydryl-containing peptide and the maleimide-activated components were reacted in a 1.5:1 molar ratio and the reaction was monitored using Ellman's assay for free sulphydryls (Thermo Fisher Scientific, Waltham, MA). Reactions were generally complete within 2 h. Excess unconjugated peptide was removed from the conjugated product using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) and deionized water as the exchange buffer. The product was aliquoted, then lyophilized and quantified.

The lyophilized conjugates were resuspended in water before use and analyzed for their PEG and amine components. PEG was quantified using the method of Sims and Snape and modified for a 96-well format [15]. Briefly, 80 μ L of sample and 20 μ L of 5% barium chloride solution (in 1 M HCl) were added sequentially to each well of a 96-well plate. 10 μ L 0.1 N iodine solution was then added before taking the absorbance readings at 535 nm ($A_{535\text{nm}}$). 10 kDa PEG (Sigma, St Louis, MO) was used as the calibration standard. Amines were quantified using the CBQCA assay kit (Invitrogen, Carlsbad, CA) with the respective peptides used to generate the standard curve.

2.2. Fibrin polymerization and degradation

Enzyme-catalyzed polymerization of mixtures of fibrinogen and GPRP_n-PEG, or control GPSP_n-PEG, conjugates was initiated in 96-well plates. Briefly, 10 μ L of the conjugate and 40 μ L of 2.5 mg/mL human fibrinogen (FIB-3; Enzyme Research Laboratories, South Bend, IN) were pre-incubated for 1 h. Following, 50 μ L of 0.5 NIH units/mL human α -thrombin and 10 Loewy units/mL human factor XIIIa (both from Enzyme Research Laboratories) were added to the fibrinogen mixture to initiate polymerization. The turbidity ($A_{350\text{nm}}$) of the mixtures was followed using a plate reader. To determine the amount of unclotted protein, the isolated clot liquor was analyzed for total soluble protein using the Quant-iT protein assay (Invitrogen). To obtain clot degradation profiles, 100 μ L 0.01 mg/mL human plasmin (Enzyme Research Laboratories) was overlaid on the 100 μ L clots in 96-well plates and agitated on the Eppendorf MixMate at 400 rpm. Turbidity readings and 5 μ L sample aliquots were taken at 1, 3, 5, 7, 9h timepoints for analysis of total soluble protein using the Quant-iT protein assay. All reactions were conducted in buffer comprising 25 mM Tris-HCl pH 7.5, 137 mM NaCl, 5 mM CaCl₂.

2.3. Crosslinking assays

Coomassie-stained SDS-PAGE gels and Western blots were used to quantitatively and qualitatively assess the extent of factor XIIIa catalyzed crosslinking in fibrin clots. Briefly, mixtures of fibrinogen and GPRP_n-PEG, or control GPSP_n-PEG, conjugates were prepared as described in the turbidity assays. The mixtures were allowed to polymerize following enzyme addition for 1 h, followed by boiling for 5 min to quench further reaction. An equal volume of 2X Laemmli buffer (Biorad, Hercules, CA) containing 5% beta-mercaptoethanol was added to the clots, which were further vortexed and boiled to ensure complete dissolution. Samples were electrophoresed on 10% SDS-PAGE gels. SimplyBlue (Invitrogen) was used for Coomassie-staining of the gels following the manufacturer's protocol. The stained gel was imaged using the Odyssey infrared imaging system (LI-COR, Lincoln, NE) in the 700 nm channel. The bands corresponding to the monomeric α , β , and γ chains on the Coomassie-stained gels were quantified using ImageJ, normalized against the non-crosslinkable β chain in each sample, then normalized against the uncrosslinked negative controls run on each gel.

For Western blots, the proteins were transferred to Immobilon-FL PVDF membranes (Millipore, Billerica, MA) overnight. Following, the membranes were blocked with IR Blocking Buffer (Rockland, Gilbertsville, PA) and probed separately for the fibrinogen α chain (primary: sc-33917 from Santa Cruz Biotechnology, Santa Cruz, CA; secondary: IRDye 800CW donkey anti-goat IgG from LI-COR) and fibrinogen γ chain (primary: sc-133157 from Santa Cruz Biotechnology; secondary: IRDye 680LT donkey anti-mouse IgG from LI-COR). The membrane was stripped between blotting by incubating with 6 M guanidine hydrochloride at 65°C until the antibody fluorescent signal was eliminated.

2.4. Rheological assays

The Bohlin CVO 120 high resolution rheometer (Malvern Instruments, Westborough, MA) with plate-plate geometry was used to assess the viscoelastic characteristics of fibrin clots at room temperature. Briefly, mixtures of fibrinogen and GPRP_n-PEG, or control GPSP_n-PEG, conjugates were prepared as described in the turbidity assays. Following the addition of thrombin and factor XIIIa, the solutions were mixed quickly by pipetting and immediately added to the bottom plate of the rheometer. The upper plate (14 mm diameter) was immediately lowered to a gap size of 1 mm and the mixture was allowed to polymerize for 1 h in a humid chamber. Following, oscillating measurements were taken over a frequency range of 0.05 to 1.0 Hz at a constant strain of 0.5%. Each condition was tested with at least 8 samples.

2.5. Confocal imaging

Fluorescently-labeled fibrinogen was prepared by conjugation to the amine-reactive Alexa Fluor® 555 succinimidyl ester (Invitrogen) following the manufacturer's recommendation. Clots containing 10% labeled fibrinogen were imaged using the Zeiss 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Mixtures of fibrinogen and GPRP_n-PEG, or control GPSP_n-PEG, conjugates were prepared as described in the turbidity assays. Following the addition of thrombin and factor XIIIa, the solutions were mixed quickly by pipetting and immediately added to a glass slide. The mixture was overlaid with a glass coverslip supported by a 0.3 mm spacer. The open ends of the chamber were sealed with nail polish and the mixture allowed to polymerize within the chamber for 1 h before imaging. A 20-section Z-stack (10 μ m slices) of the 89×89 μ m field under the 100× objective was captured for reconstruction of the 3D network structure. At least 3 clots were formed independently for each condition, with 4 random samplings taken per clot.

2.6. Statistical Analysis

All graphs and analyses were prepared in GraphPad PRISM 5.0. Results are presented as mean $\pm$ SD. Statistical comparisons for conjugate titration sets were based on one-way ANOVA using the Tukey post-hoc test for pair-wise comparisons with significance defined by $p < 0.05$. Comparisons of conjugates and their paired controls (for crosslinking and rheological data) were made using the Student's t-test.

3. Results

3.1. Characterizing fibrin clot formation

To establish the basic analytical parameters for fibrin clot formation, we assessed the clotting characteristics of fibrinogen in the presence of the control materials – peptides GPRP and GPSP, and non-functionalized PEG. As expected, the presence of the peptide GPRP reduced clot turbidity in a dose-dependent manner (Fig. 2A), corroborating the early findings of Doolittle et al. [8]. In contrast, our control peptide, GPSP, did not significantly impact the turbidity profile of the clotting mixture at the corresponding dosages (Fig. 2B).

Interestingly, the presence of large amounts (100:1 molar ratio PEG-to-fibrinogen) of 10 kDa PEG amplified the turbidity of the clotting mixtures (Fig. 2C). To further investigate this phenomenon, the initial (solid symbols) and final (open symbols) turbidity values of the different mixtures were plotted (Fig. 2D), indicating that the presence of PEG increased the final absorbance but not the initial absorbance of the mixture, despite the 1 h incubation step prior to the addition of clotting enzymes, thrombin and factor XIIIa.

The clotting half-time, defined as the time required for the clot to reach half the final turbidity value, was calculated from the clotting curves (Fig. 2E). The results indicate that a 100:1 molar excess of GPRP not only reduced the overall turbidity but also increased the clotting half-time of the mixture. While PEG significantly increased mixture turbidity, it did not have a statistically significant impact on clotting half-time.

The fibrin clot was then carefully compressed and the concentration of soluble protein in the liquid fraction (i.e. clot liquor) was assessed. The amount of clotted protein was back-calculated using the respective controls and reported as % clottable protein (Fig. 2F). As expected, the results indicate that GPRP, but not the control GPSP, reduced the % clottable protein of the mixtures. Intriguingly, a 100:1 excess of PEG of the mixture had no significant effect on the overall clottability of the mixture despite the increased turbidity of the resulting clot. Further experiments carried out with the peptide-PEG conjugates were therefore limited to conjugate-to-fibrinogen molar ratios of 10:1 and below.

3.2. Mixture clottability and clotting half-time

Pairs of GPRP_n-PEG and GPSP_n-PEG conjugates were prepared using the well-characterized sulfhydryl-maleimide chemistry (refer to supplementary data, Table S1, for characterization data). The effect of these conjugates on fibrin clot formation was assessed at 1:10, 1:1 and 10:1 conjugate-to-fibrinogen molar concentrations. Clots were formed at all three dosages for all the conjugates evaluated in this work. However, the tenuous clots formed at the highest GPRP_n-PEG dosages (open symbols/dashed lines), i.e. 10:1, had a comparatively low clottability of 70-80% compared to their respective GPSP_n-PEG controls (solid symbols/solid lines) with clottability above 90% (Fig. 3A, B). Moreover, the clotting half-times of these mixtures were significantly longer than the controls (Fig. 3C, D). At a 1:1 ratio, the clotting half-times for the GPRP₂-PEG conjugates were not significantly perturbed and the overall turbidity profiles were similar in shape to that of the controls (refer to supplementary data, Figure S2). A similar phenomenon was observed for the 2 kDa GPRP₄-PEG conjugate. In contrast, the profiles for the 10 and 20 kDa GPRP₄-PEG conjugates were much flatter, nearly doubling the apparent clotting half-time.

3.3. Turbidity measurements

A closer examination of the turbidity profiles indicate that the final absorbances of the mixtures at a 10:1 conjugate-to-fibrinogen ratio were consistently lower than the controls, possibly indicative of both reduced overall clottability and structural changes due to the increased clotting half-time. A notable result was observed at a 1:1 ratio, with the 2 kDa GPRP₂-PEG conjugate increasing the final turbidity, and the 5 and 7.5 kDa GPRP₂-PEG conjugates reducing the overall turbidity (Fig. 4A). Similarly, the presence of the 2, 10 and 20 kDa GPRP₄-PEG conjugates at a 1:10 ratio slightly increased the overall absorbance of the mixtures although the opposite phenomenon was observed at the 1:1 and 10:1 conjugate-to-fibrinogen ratios (Fig. 4B).

A plot of initial mixture turbidities prior to the addition of clotting enzymes showed that the pre-incubation of the GPRP_n-PEG conjugates with fibrinogen generally had the greatest impact on mixture turbidity at a 1:1 ratio (Fig. 4C, D). This trend was not observed for the

corresponding GPSP_n-PEG controls. The increased light scattering at this particular ratio is suggestive of fibrinogen assembly arising from knob:hole interactions since each fibrinogen has up to 4 available holes for binding to the 2-arm and 4-arm conjugates. Further experiments were therefore pursued using a 1:1 conjugate-to-fibrinogen molar concentration.

3.4. Factor XIIIa-mediated crosslinking

As clot turbidity reflects differences in fibril organization in clotting mixtures, the presence of GPRP_n-PEG conjugates may also perturb the crosslinking pattern of the fibrin(ogen) chains within the clot. Surprisingly, the GPRP_n-PEG conjugates appeared to reduce the extent of α chain crosslinking compared to their respective GPSP_n-PEG controls, particularly in the case of the 3.5 kDa GPRP₂-PEG and 20 kDa GPRP₄-PEG (Fig. 5A). In contrast, the extent of γ chain crosslinking was not perturbed by the presence of peptide-PEG conjugates under the clotting conditions used (Fig. 5B). The presence of unconjugated peptide (denoted GPxP) did not perturb the extent of α and γ chain crosslinking.

For further clarification of these results, identical experiments were run in the absence of thrombin and with a large excess of factor XIIIa (50 Loewy units/mL) to compensate for its reduced activity in the absence of thrombin. As expected, the results indicate that the presence of the GPRP_n-PEG conjugates increased the extent of both α and γ chain crosslinking, notably in the presence of the 10 kDa GPRP₄-PEG conjugate (Fig. 5C, D). The trend in the % crosslinked chains suggests a size dependency wherein the optimum PEG size for promoting factor XIIIa crosslinking in the absence of thrombin is around 3.5 kDa for GPRP₂-PEG and 10 kDa for GPRP₄-PEG. These particular conjugates were found to reduce the % α chain crosslinked in the presence of thrombin while smaller conjugates such as the 2 kDa GPRP₂-PEG and 2 kDa GPRP₄-PEG did not significantly perturb the extent of crosslinking of both α and γ chains. Interestingly, Western blots for the α and γ chains did not suggest any striking differences in the crosslinking pattern obtained in the presence of the conjugates as compared to the controls (refer to supplementary data Figure S3).

3.5. Confocal imaging

For a more direct evaluation of the type of network structural changes imposed by the presence of the GPRP_n-PEG conjugates, clots were formed on glass slides using Alexa Fluor 555-labelled fibrinogen to enable direct visualization and imaging using confocal microscopy. Using 10% labeled fibrinogen with a final fibrinogen concentration of 1 mg/mL, network formation was clearly visible under control conditions (Fig. 6A). In the presence of a 100-molar excess of non-functionalized PEG, where mixture turbidity was significantly increased (Fig. 2C), the fibers were thicker and more clearly defined as compared to the control (Fig. 6B). Conversely, at a 10:1 peptide-to-fibrinogen molar dosing, a more fine-meshed network was formed in the presence of GPRP (Fig. 6C) as compared to GPSP (Fig. 6D). The noisy background appears to be an artifact arising from the higher osmolarity of these mixtures due to the added peptide.

Focusing on the network structure of clots formed in the presence of an equimolar dosing of peptide-PEG conjugate, it was noted that clots formed in the presence of the GPSP_n-PEG conjugates were very similar to the control (compare Fig. 6A with 6F, H, J, L, N, P, R), while very clear conjugate size-dependent trends were observed with the GPRP_n-PEG conjugates. Notably, the fibers formed in the presence of 2 kDa GPRP₂-PEG (Fig. 6E) and 2 kDa GPRP₄-PEG (Fig. 6M) appeared to be thicker and straighter. The networks formed in the presence of 3.5 kDa and 5 kDa GPRP₂-PEG (Fig. 6G, I) appeared to resemble their respective GPSP₂-PEG controls (Fig. 6H, J) the closest, but with the inclusion of tight nodules of proteins dispersed throughout the matrix. In the presence of the larger GPRP_n-

PEG conjugates – 7.5 kDa GPRP₂-PEG, 10 kDa and 20 kDa GPRP₄-PEG – the networks appeared to comprise ultrathin and highly-branched fibers nearing the resolution limits of the microscope (Fig. 6K, O, Q). These results corroborate the significantly decreased clot turbidities of these mixtures despite their maintained clottability as determined by clottable protein measurements. Tight nodules of protein continued to be visible in these matrices, but not in their respective controls (Fig. 6L, P, R), suggesting that these are clusters of GPRP_n-PEG-bound fibrin(ogen).

3.6. Rheological characterization

Utilizing the same clot preparation protocol, the rheological properties of these matrices were assessed using a parallel-plate setup in a constant-stress rheometer. The results suggest that the presence of the GPRP_n-PEG conjugates generally reduces the elastic modulus of the clot although the loss tangent, reflecting the clot viscosity, was not significantly perturbed (Fig. 7). Intriguingly, this trend in elastic moduli appears to resemble the extent of α chain crosslinking (Fig. 5A), despite the vast differences in network structure observed through confocal imaging (Fig. 6), suggesting that clot mechanical stability under our experimental conditions is more dependent upon α chain crosslinking than network structure.

3.7. Clot degradation

Clots formed in the presence of peptide-PEG conjugates were overlaid with plasmin and incubated on a shaker incubator at room temperature. The results suggest that all the clots degraded at a constant rate such that the mixture turned clear in approximately 9 hours, regardless of the starting turbidity of the clot (Fig. 8A,B). For a more objective comparison of the degradation rate across the different mixtures, the amount of soluble protein in the overlay was assessed. Interestingly, the results indicate that the degradation rate of the clots did not vary significantly across the different conjugates (Fig. 8C,D). Similar results were obtained for clots formed in the presence of varying doses of the conjugates (data not shown).

4. Discussion

Past attempts have been made to alter fibrin matrix properties through the direct conjugation of PEG to fibrinogen, followed by UV-induced crosslinking of the functionalized PEG moieties, or the addition of thrombin to induce gelation [16-18]. In contrast, the objective of the present studies was to explore the possibility of altering fibrin network architecture through the use of multivalent peptide-PEG conjugates that actively engage fibrin polymerization holes, potentially facilitating alternative mechanisms of polymerization. In this work, we have identified various techniques of analyzing fibrin hydrogels and successfully demonstrated the ability to modify fibrin network structure through the addition of GPRP₂-PEG and GPRP₄-PEG conjugates.

It has been known for decades that fibrin clot structure can be inferred from turbidity profiles of clotting mixtures of fibrinogen and thrombin [7]. In general, thicker fibrin bundles are associated with turbid clots whereas thinner fibrin bundles result in clear or glassy clots. A clear mixture can also be indicative of the absence of fibril assembly and clot formation, such as in the presence of large excess of knob A mimics, notably the peptide GPRP in this paper. The use of real-time turbidity measurements of clotting mixtures in conjunction with end-point protein measurements allowed a rapid screen of clotting mixtures to identify mixture compositions that perturb, but not completely inhibit, the polymerization process. As expected, mixtures having a 1:1 conjugate-to-fibrinogen dosing were identified as having the greatest potential impact on fibrin matrix structure. This is suggestive of the formation of a hybrid assembly wherein each peptide-PEG conjugate

interacts with up to 4 fibrinogen molecules depending on conjugate valency, while each fibrinogen molecule has 4 holes that may participate in knob:hole interactions.

By varying the size and valency of the peptide-PEG conjugate added to fibrinogen, we were able to induce significant changes in fibrin network structure without sacrificing overall clottability of the mixture. In particular, the results indicate that the larger conjugates used in this study favored the formation of fine highly branched networks, even as fibrinogen, conjugate, thrombin and factor XIIIa molar concentrations were kept constant (Fig. 6). Moreover, the networks formed were stable and did not appear to change following an overnight incubation (data not shown). This suggests that larger conjugates with long mobile PEG chain linkers between the peptide knobs were interfering with the lateral aggregation of the protofibrils to form thick fibers, possibly by non-directional orienting of fibrinogen molecules mediated by knob:pocket interactions prior to thrombin addition. Intriguingly, such networks are reminiscent of those formed in the presence of high thrombin concentrations, where rapid fibrinopeptide removal and formation of knob:hole interactions results in a fine-meshed but characteristically weaker clot [6, 19]. These results suggest that the design of bisvalent or double-pincer peptide-PEG linkers could favor the formation of coarse networks with thick fibers by pinning the ends of the fibrinogen molecule together to form protofibril-like structures, even in the absence of clotting enzymes.

It has been shown that the crosslinking activity of factor XIIIa is heavily influenced by fibrin assembly and fiber orientation [6, 13, 20]. This is supported by our results obtained in the absence of thrombin whereby the juxtaposition of crosslinking sites via knob:hole interactions is mediated by the peptide-PEG conjugate in lieu of thrombin-cleaved fibrin α and β chains (Fig. 5). Interestingly, despite the changes in gross network structure obtained in the presence of the conjugates, we did not observe large changes in the extent of crosslinking in these clots in the presence of thrombin, an observation supported by rheological assessment of the hydrogels (Fig. 7) as factor XIIIa crosslinking is also associated with clot stiffness [6, 21]. Nonetheless, the trend appears to suggest a decrease in the extent of α chain crosslinking in the presence of all the conjugates, with the exception of the 2 kDa GPRP₂-PEG and 2 kDa GPRP₄-PEG conjugates. As α chain crosslinking is associated with the lateral aggregation of fibers, this again suggests that the large peptide-PEG conjugates are inhibiting the lateral aggregation of the fibrils and that alternative conjugate architectures may be designed to augment factor XIIIa crosslinking and clot stiffness.

We also carried out degradation assays using an overlay of plasmin on the clot. While the results did not suggest any significant differences between the clots (Fig. 8), we note that the rate of plasmin-induced degradation reflects several parameters, including the diffusion of plasmin into the clot and fiber susceptibility to plasmin degradation. Altering fibrin network structure via fibrinogen and thrombin concentration, early studies have suggested that thin fibers are more resistant to plasmin digestion than thick fibers [22]. While it is now generally accepted that thin fibers are cleaved at a faster rate than thick fibers, the relationship between fibrin network structure and fibrinolysis, whether intrinsically or extrinsically induced, is hardly direct [23]. Nonetheless, these results suggest that the proposed non-covalent method of perturbing fibrin clot structure does not affect the degradability of the resulting matrix.

5. Conclusion

This work demonstrates the feasibility of modulating fibrin matrix structure through the perturbation of knob:hole interactions via peptide-PEG conjugates. Notably, an equimolar supplementation of the conjugate is sufficient to mediate a myriad of changes in fibrin

network structure, even though this work was confined to simple 2-arm and 4-arm PEG architectures ranging from 2 to 20 kDa in size. Clinically relevant parameters such as clotting and degradation rates were not significantly altered at the conditions used, suggesting that such non-covalent means of modifying network structure has the potential to allow the independent manipulation of biochemical and mechanical cues. This will enable the deliberate design of peptide-PEG conjugates for creating fibrin-PEG hybrid gels with enhanced usability in tissue engineering as well as surgical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Fibrin polymerization schematic. (A) Steps involved in native fibrin polymerization. (B) Proposed modification to fibrin polymerization in the presence of GPRP_n-PEG conjugates. Fibrinogen Aα chain in green, Bβ chain in blue, γ chain in red. Peptide-PEG conjugates in black.

Fig. 2.

Fibrin polymerization parameters (using 1 mg/mL fibrinogen, 0.25 NIH U/mL thrombin, 5 Loewy units/mL factor XIIIa) in the presence of GPRP, GPSP, and PEG. Representative turbidity profiles in the presence of GPRP (A), GPSP (B) and PEG (C) at 1:1, 10:1 and 100:1 peptide/PEG-to-fibrinogen molar ratios. (D) Plot of absolute initial (solid symbols) turbidity values and final (empty symbols) turbidity values from the entire experimental data set ($n = 6$). (E) Clotting half-times, defined as the time required for the clot to reach half the final turbidity value (at 1 h following enzyme addition), calculated from the turbidity curves. (F) The percentage of clottable protein, back-calculated from the amount of soluble protein in the clot liquor extracted from compressed clots, normalized against controls without clotting enzymes.

Fig. 3.

Fibrin polymerization parameters in the presence of peptide-PEG conjugates. % Clottable protein of hydrogels formed in the presence of GPxP₂-PEG (A) and GPxP₄-PEG conjugates (B) at 1:10, 1:1 and 10:1 conjugate-to-fibrinogen molar ratios. Clotting half-times in the presence of GPxP₂-PEG (C) and GPxP₄-PEG conjugates (D). Solid symbols and lines correspond to data for the GPSP_n-PEG control conjugates; empty symbols and dashed lines correspond to data for the GPRP_n-PEG conjugates.

Fig. 4.

Turbidity of hydrogels formed in the presence of peptide-PEG conjugates. Final turbidity values of hydrogels formed in the presence of GPxP₂-PEG (A) and GPxP₄-PEG conjugates (B) at 1:10, 1:1 and 10:1 conjugate-to-fibrinogen molar ratios. Initial turbidity values of hydrogels formed in the presence of GPxP₂-PEG (C) and GPxP₄-PEG conjugates (D). Solid symbols and lines correspond to data for the GPSP_n-PEG control conjugates; empty symbols and dashed lines correspond to data for the GPRP_n-PEG conjugates.

Fig. 5.

Densitometry analysis of fibrin(ogen) chain species in hydrogels formed in the presence of peptide-PEG conjugates at a 1:1 conjugate-to-fibrinogen molar ratio. Bands corresponding to α , β , and γ chains on the Coomassie-stained gels were quantified using ImageJ, normalized against the non-crosslinkable β chain in each sample, then normalized against the uncrosslinked negative controls run on each gel. % crosslinked α chain (A) and γ chain (B) in hydrogels formed in the presence of both thrombin and factor XIIIa. % crosslinked α chain (C) and γ chain (D) in hydrogels formed in the presence of 50 Loewy units/mL factor XIIIa only. Indicated statistical significance calculated from two-tailed t-tests of GPRP_n-PEG conjugates against their respective GPSP_n-PEG controls ($n \geq 6$).

Fig. 6.

Confocal images of hydrogels. Hydrogels were formed within sealed chambers on glass slides and imaged using laser scanning confocal microscopy. Images were rendered from $45 \times 45 \times 10 \mu\text{m}$ slices. (A) Control clot (1 mg/mL fibrinogen, 0.25 NIH U/mL thrombin, 5 Loewy units/mL factor XIIIa). (B) Representative network structure formed in the presence of 100:1 molar ratio of PEG-to-fibrinogen. (C) Network structure in the presence of 10:1 molar ratio of GPRP-to-fibrinogen. (D) Network structure in the presence of 10:1 molar ratio of GPSP-to-fibrinogen. Subsequent paired images are of hydrogels formed in the presence of GPRP_n-PEG or GPSP_n-PEG conjugates at a 1:1 conjugate:fibrinogen molar ratio as follows: 2 kDa GPxP₂-PEG (E,F), 3.5 kDa GPxP₂-PEG (G,H), 5 kDa GPxP₂-PEG (I,J), 7.5 kDa GPxP₂-PEG (K,L), 2 kDa GPxP₄-PEG (M,N), 10 kDa GPxP₄-PEG (O,P), 20 kDa GPxP₄-PEG (Q,R).

Fig. 7. Rheological characterization of hydrogels formed in the presence of peptide-PEG conjugates at a 1:1 conjugate:fibrinogen molar ratio. Elastic moduli (A) and loss tangents (B) were obtained from a sample size of at least $n = 6$ for each condition. Indicated statistical significance calculated from two-tailed t-tests of GPRP_n-PEG conjugates against their respective GPSP_n-PEG controls.

Fig. 8.

Degradation profiles of hydrogels formed in the presence of peptide-PEG conjugates at a 1:1 conjugate-to-fibrinogen molar ratio upon the addition of an equal volume of 0.01 mg/mL ($\sim 0.06 \mu\text{mol/min/mL}$) plasmin. Change in turbidity of hydrogels formed in the presence of GPxP₂-PEG (A) and GPxP₄-PEG conjugates (B). Quantitation of soluble protein in the plasmin overlay of hydrogels formed in the presence of GPxP₂-PEG (C) and GPxP₄-PEG conjugates (D).