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Supplemental Material:
Fluorescently Conjugated Annular Fibrin Clot for Multiplexed Real-time Digestion Analysis

Ziqian Zeng [a,b], Tanmaye Nallan Chakravarthula [a,b], Charanya Muralidharan [c,d], Abigail Hall [a], Amelia K. Linnemann [c,d], Nathan J. Alves [a,b,c*]

[a] Department of Emergency Medicine, Indiana University School of Medicine, Indianapolis, IN
[b] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN
[c] Departments of Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, IN
[d] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN

[*] Corresponding author:
Nathan J. Alves, PhD
Indiana University School of Medicine
635 Barnhill Dr. Rm. 2063
Indianapolis, IN 46202, United States of America
E-mail address: nalves@iu.edu (N.J. Alves).

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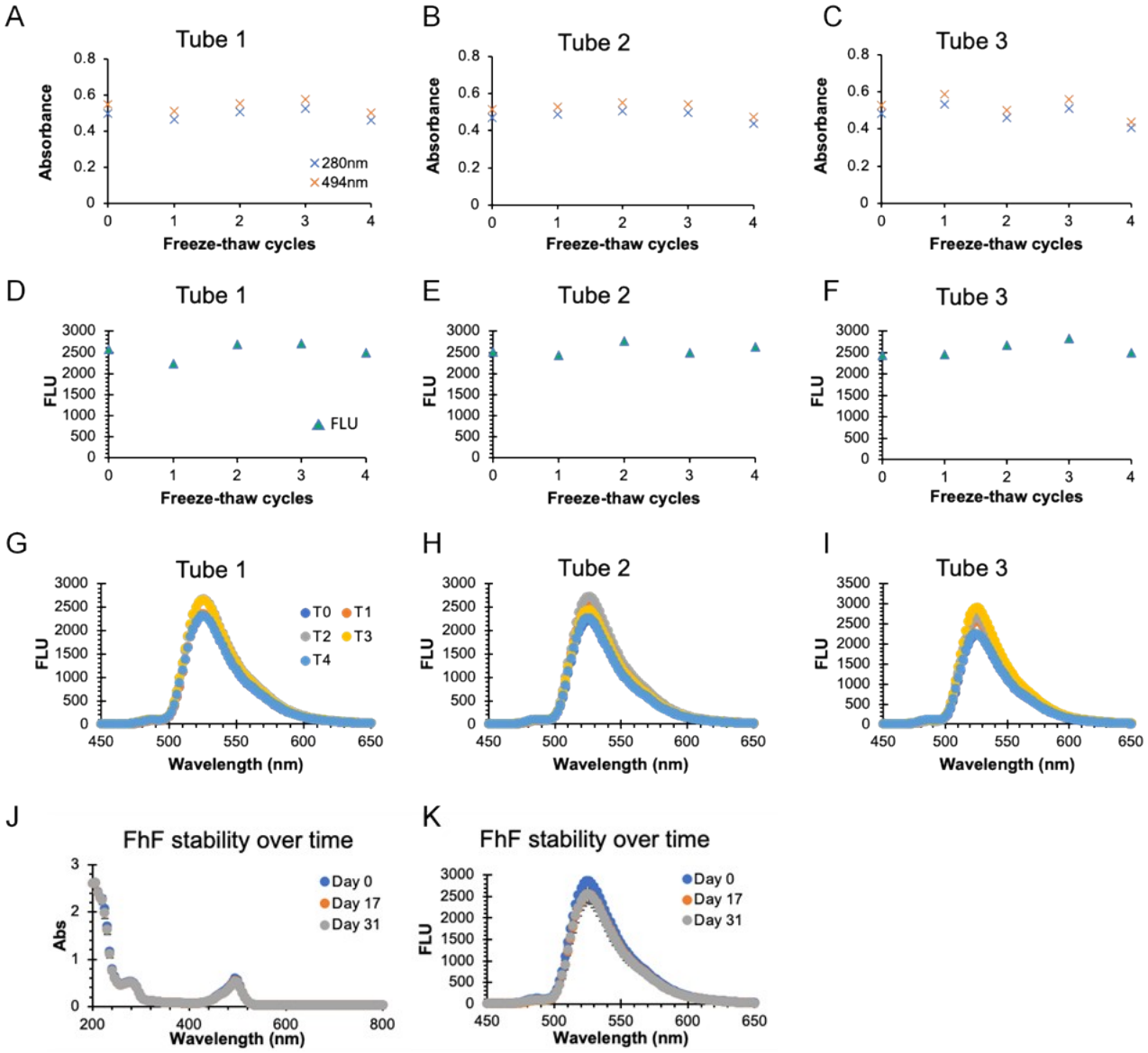
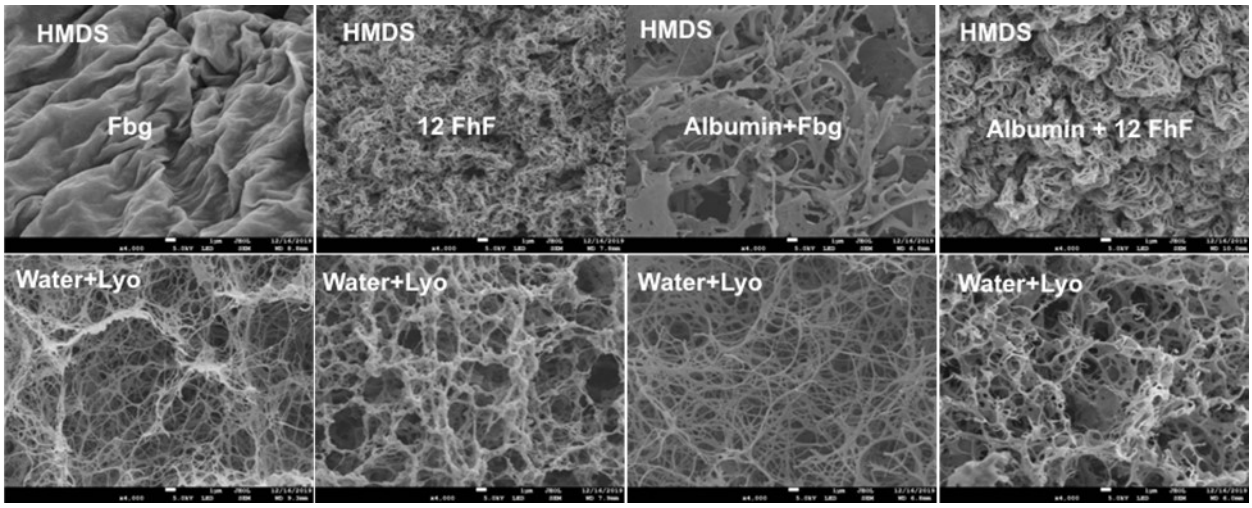
Suppl. Table.1 Total pore area and fiber diameter of clot at different fibrinogen to 12FhF ratios

Clot formula	Pure 12FhF	12FhF R5	12FhF R10	12FhF R30	12FhF R50	Fibrinogen
Fiber diameter (nm)	***165 ± 115	***153 ± 83	***157 ± 114	*125 ± 79	111 ± 78	105 ± 59
Pore size (μm^2)	***0.5 ± 0.8	***0.9 ± 1.6	***1.1 ± 2.1	*1.3 ± 3.0	1.6 ± 3.4	1.7 ± 3.3
Total pore area	***5.7% ± 2.7%	*24.5% ± 7.5%	**28.0% ± 1.7%	39.2% ± 6.3%	39.3% ± 0.9%	40.4% ± 4.5%

Note: P values of groups comparing to the fibrinogen control group were determined by two-tailed unpaired t test, *** denotes P value <0.0001, ** denotes P value <0.001, * denotes P value <0.05.



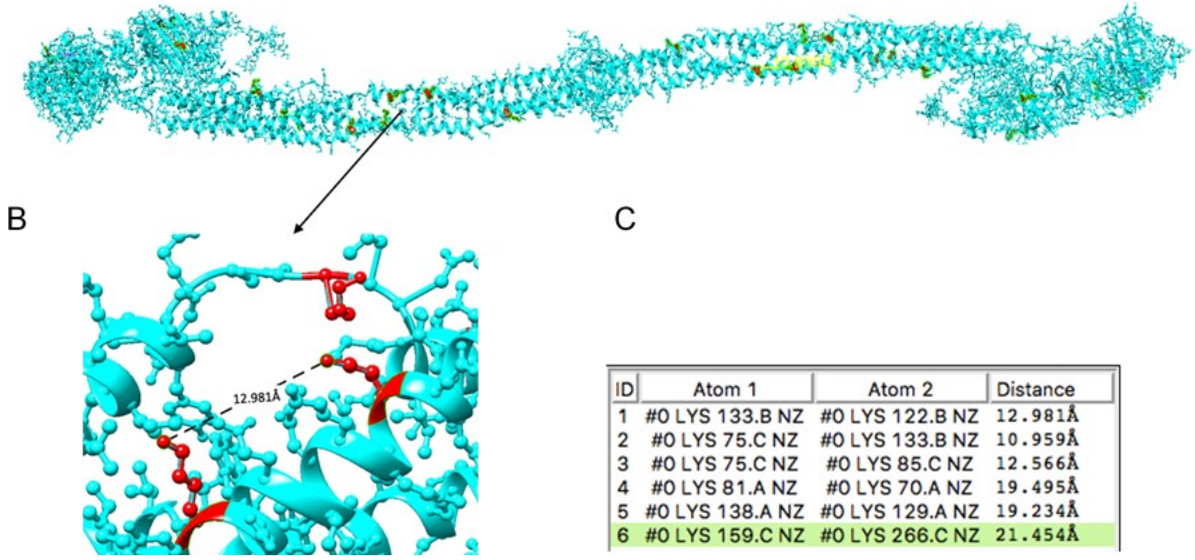
Suppl. Fig. 1: 12FITC-Fibrinogen (FhF) molecular and fluorescent stability after 4 freeze-thaw cycles. Three tubes of product were examined at (A, B, C) absorbance 280nm and 494 nm of stock aliquots at 20-fold dilution, (D, E, F) fluorescence endpoint Ex 495nm and Em 519 nm of stock aliquots at 400-fold dilution, and (G, H, I) additional fluorescent emission spectrum (Ex 495 nm, Em 450 - 650 nm, 400-fold dilution). No significant difference was found for product absorbance after 3 cycles (n=3, p_280 nm=0.1089, p_494nm=0.0897) and no significant difference were observed for emission spectrum as well as fluorescence endpoint after 4 cycles (n=3, p=0.2312). In addition, FhF aliquots were examined over storage time using the same metric. Both (J) absorbance spectrum and (K) fluorescence (Ex 495 nm, Em 450 - 650 nm, 400-fold dilution) showed consistent values over 31 days. This result indicates fluorescence intensity of FITC-Fbg product is stable after vigorous freeze-thaw cycles and storage.



Suppl. Fig. 2: Representative SEM images (4,000X) were compared for ethanol-Bis(trimethylsilyl)amine (HMDS, Electron Microscopy Sciences Supplier, Hatfield, PA) (first row) and water-lyophilization dehydration methods (second row) of fibrin clots, which were formed by neat fibrinogen, neat 12FhF, Albumin + fibrinogen, and albumin + 12FhF. The HMDS method requires the washing of clot samples using a series of ethanol swaps at 30%, 50%, 70% and 100% (3 times), followed by a 50:50 ethanol to HMDS swap, and an overnight storage in 100% HMDS. However, the HMDS method results in clots that have 95% shrinkage while the lyophilization method offers a puffy dehydrated clot. Scale bar was shown as 1 μm in all images.

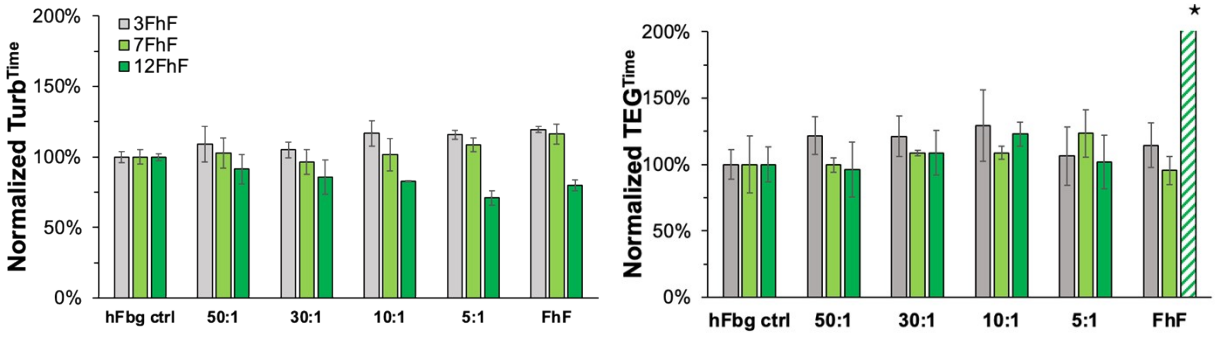
Suppl. Fig. 3: Identification of lysine / FITC conjugation sites on fibrinogen alpha, beta and gamma chains using mass spectrometry. One fibrinogen molecule has a pair of alpha, beta, and gamma chains. Available fibrinogen crystal structures (PDB entity sequence: 3GHG) are derived from the protein data bank for reference. The detailed protocol for fabricating FITC tagged fibrinogen molecules: Reacting human fibrinogen with a 200-fold excess FITC at RT for 24 hours and incubating for another 6 hours with the addition of doubled moles of fresh FITC to maximize the FITC conjugation on fibrinogen. The FITC-fibrinogen was then purified using 100kDa cutoff filter under 6 cycles of centrifugation to remove free FITC molecules. An average of 16 FITC per fibrinogen were identified through absorbance readings using the spectrometer method detailed in the main text. FITC-fibrinogen was then denatured in 8 M urea and digested by 1 µg trypsin/Lys-C protease mix for 4 hours at 37 C before dilution to 2 M urea overnight. Peptide sequences were examined using mass spectrometry. Data were subjected to post-translational modification (PTM) analysis using PEAKS Xpro to find FITC-lysine conjugation sites. Overall, PEAKS Xpro provided ~90% coverage of the fibrinogen protein and peptide sequences were included for analysis at less than 1% false discovery rate. The sum of all lysine residues in each pair are 43+36+34=113; wherein, mass spec covered lysine sites are 38+28+32=98, crystal structure known lysine sites are 16+4+5=25. Mass spec covered FITC sites are 16+6+5=27, and crystal structure known FITC sites are 4+4+5=13. Specifically, locations of these lysine are identified. Alpha chain (81% coverage, 16 sites): K89, K100, K148, K157, K227, K243, K322, K432, K437, K448, K467, K476, K558, K575, K602, K620. K238 is removed (since it had 390, but with a substitution in the peptide); Beta chain (83% coverage, 6 sites): K52, K77, K152, K163, K353, K367. K264 (missing 390 signatures though) and K426 (not full sequencing fragmentation) are removed; Gamma chain (90% coverage, 5 sites): K84, K101, K111, K146, K188. K199 is removed (missing 390, peptide has an AA substitution). Due to the lack of 100% coverage in both mass spec and available crystal structure a comprehensive identification of dominant lysine conjugation sites could not be further characterized. This data is, however; useful in demonstrating the diversity of conjugation sites indicating that there is some degree of conjugation variation even at saturating levels of FITC associated with a lysine conjugation technique of this nature.

A

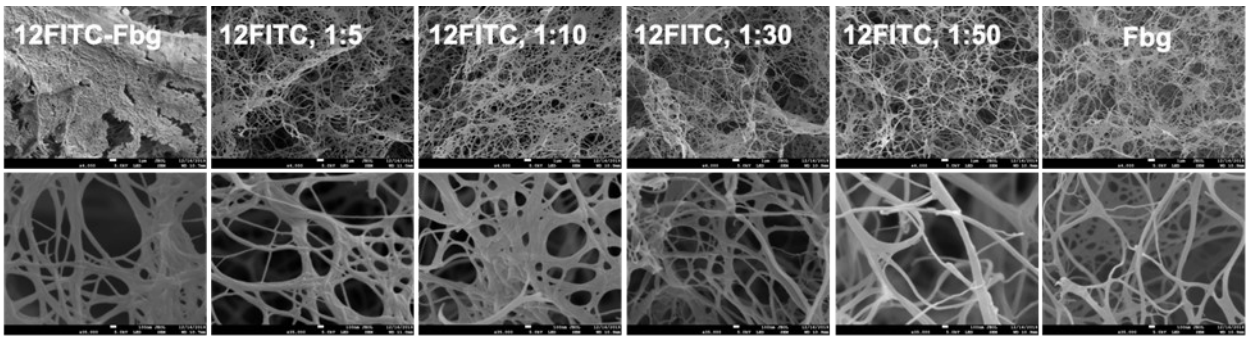


ID	Atom 1	Atom 2	Distance
1	#0 LYS 133.B NZ	#0 LYS 122.B NZ	12.981Å
2	#0 LYS 75.C NZ	#0 LYS 133.B NZ	10.959Å
3	#0 LYS 75.C NZ	#0 LYS 85.C NZ	12.566Å
4	#0 LYS 81.A NZ	#0 LYS 70.A NZ	19.495Å
5	#0 LYS 138.A NZ	#0 LYS 129.A NZ	19.234Å
6	#0 LYS 159.C NZ	#0 LYS 266.C NZ	21.454Å

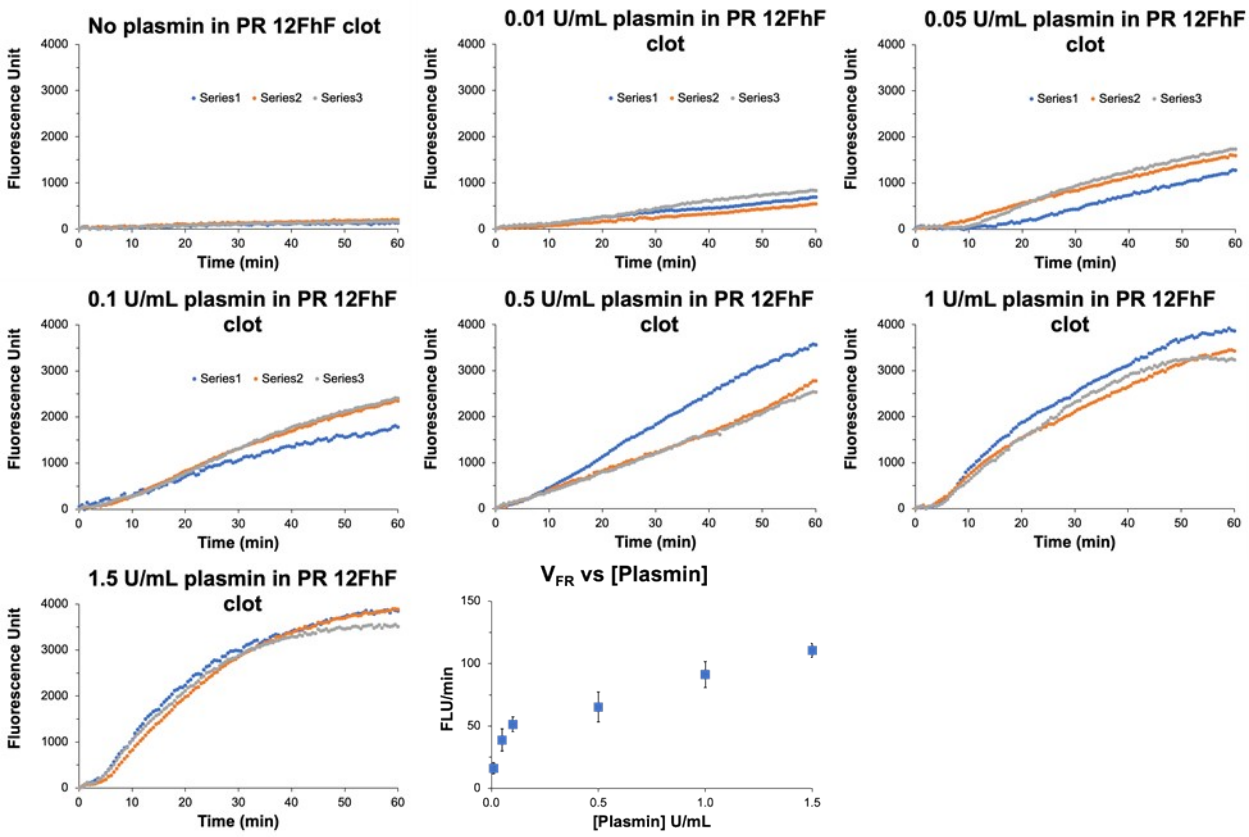
Suppl. Fig. 4: (A) Fibrinogen crystal structure (PDB entity sequence: 3GHG, protein data bank) is shown in blue ribbon with identified FITC conjugation sites labeled in red. Despite an average of 16 FITC per fibrinogen calculated via absorbance, 54 lysine sites (27 sites in one set of fibrinogen alpha, beta, and gamma chain) were found to be accessible for FITC conjugation. This indicates that the conjugation reaction yields a heterogeneous FITC-fibrinogen mixture which can have different FITC amounts per fibrinogen or are products that have similar FITC amounts per fibrinogen but possess different combinations of FITC conjugation sites. The average 16 FITC per fibrinogen is most likely contributed by both scenarios. This is typical of non-site-specific conjugation techniques such as lysine-based labeling used here. In addition, only 52.8% of the fibrinogen crystal structure is known with the unknown part mostly belonging to the alpha C domain (representing 28 more possible FITC accessible lysine sites for conjugation). (B) shows a representative example of measuring the distance of adjacent FITC conjugation sites on available fibrinogen crystal structures using Chimera. In the known crystal structure of one set of the fibrinogen chains, 6 pairs (C) totaling 10 non-repeated conjugation sites were found to be within 20 angstroms of one another. Due to the proximity of these sites, the conjugation of one site within a pair likely reduces the probability of FITC subsequently conjugating the other one due to steric effects.



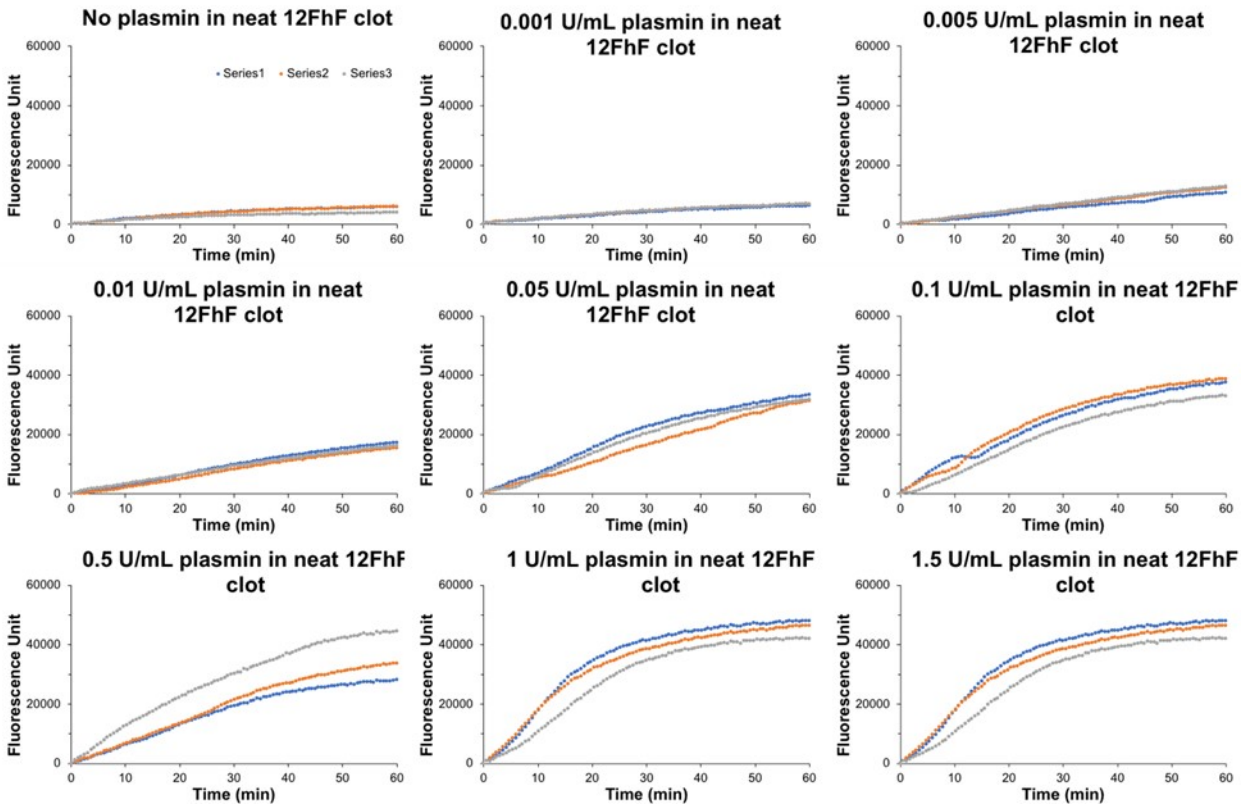
Suppl. Fig. 5: Comparing (A) $Turb^{Time}$ and (B) TEG^{Time} of FITC labeled human fibrin clots. Data were normalized by values of human fibrinogen control groups. ⋆ TEG^{Time} of neat 12-FHF is at 303%.



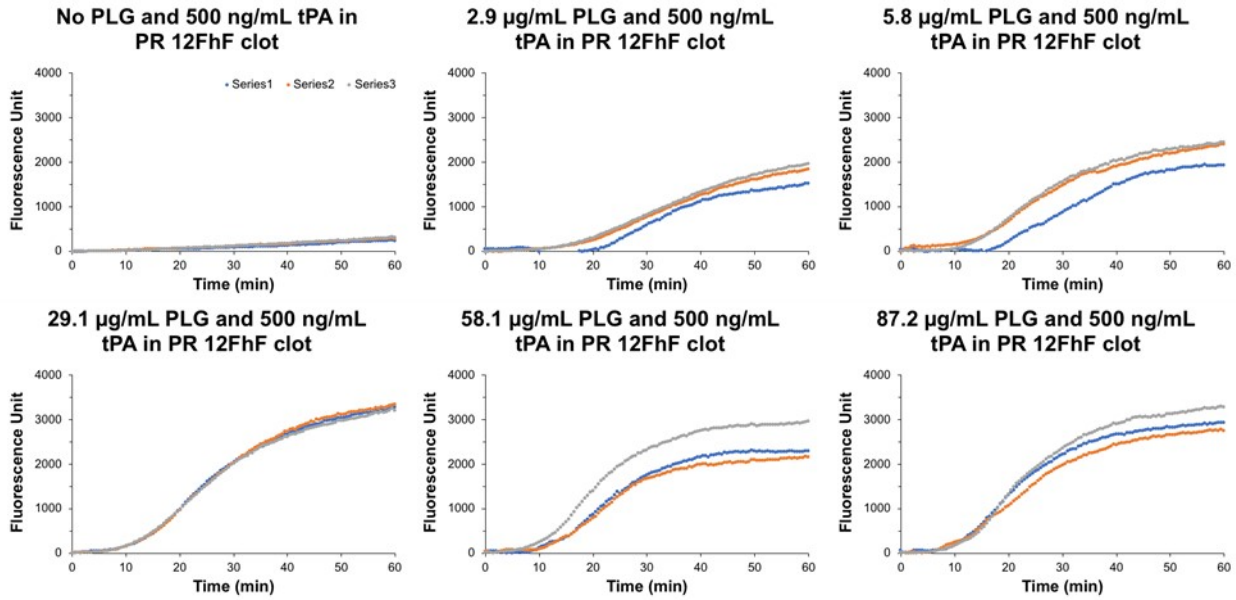
Suppl. Fig. 6: Representative SEM images of varying 12 FhF in fibrin. 4,000X (first row) and 35,000X (second row). Scale bars shown as 1 μm (first row) and 100 nm (second row).



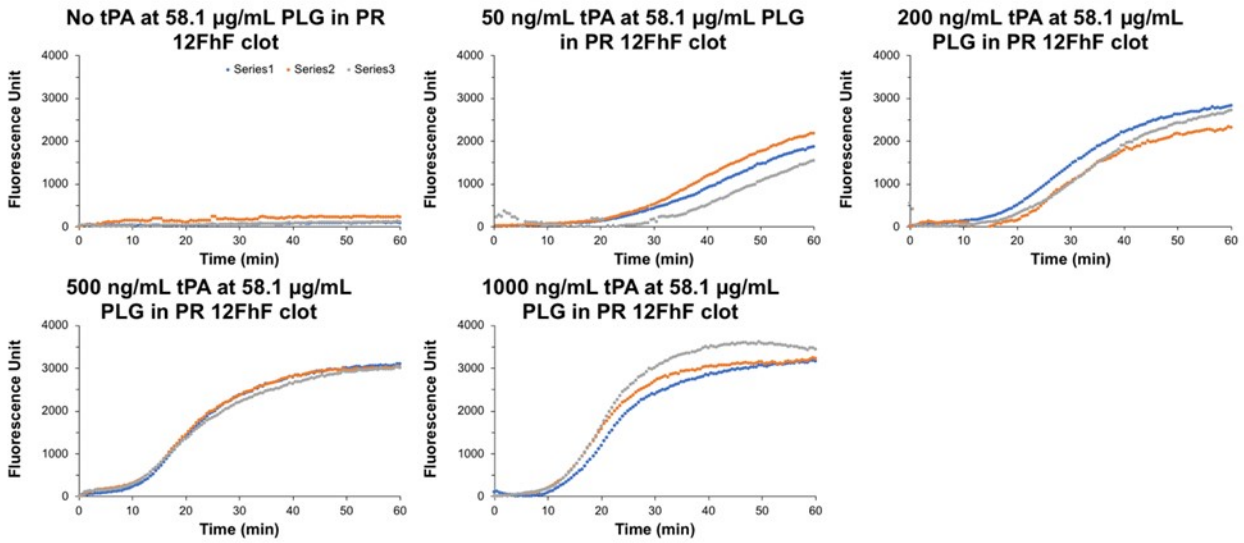
Suppl. Fig. 7: Fluorescence tracing curves of varying plasmin concentrations for digesting physiologically relevant 12FhF annular clots at 37 °C. The last figure shows V_{FR} (fluorescence release rate) over plasmin concentrations.



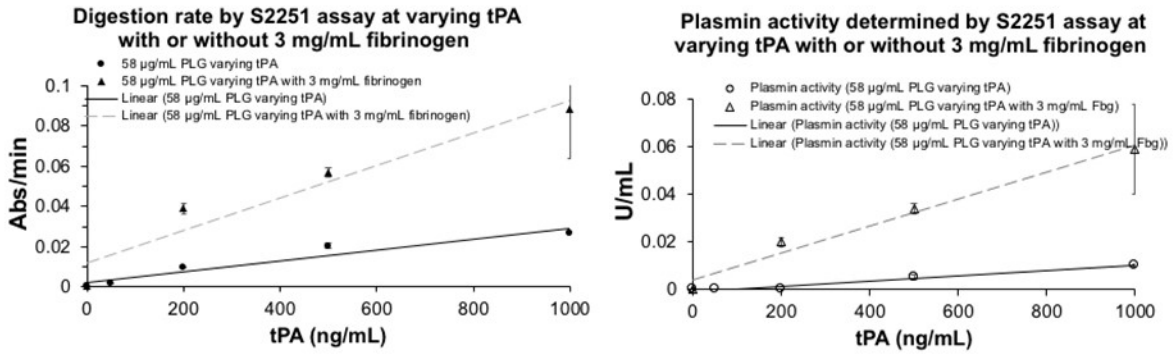
Suppl. Fig. 8: Fluorescence tracing curves of varying plasmin concentrations for digesting neat 12FhF annular clots at 37 °C.



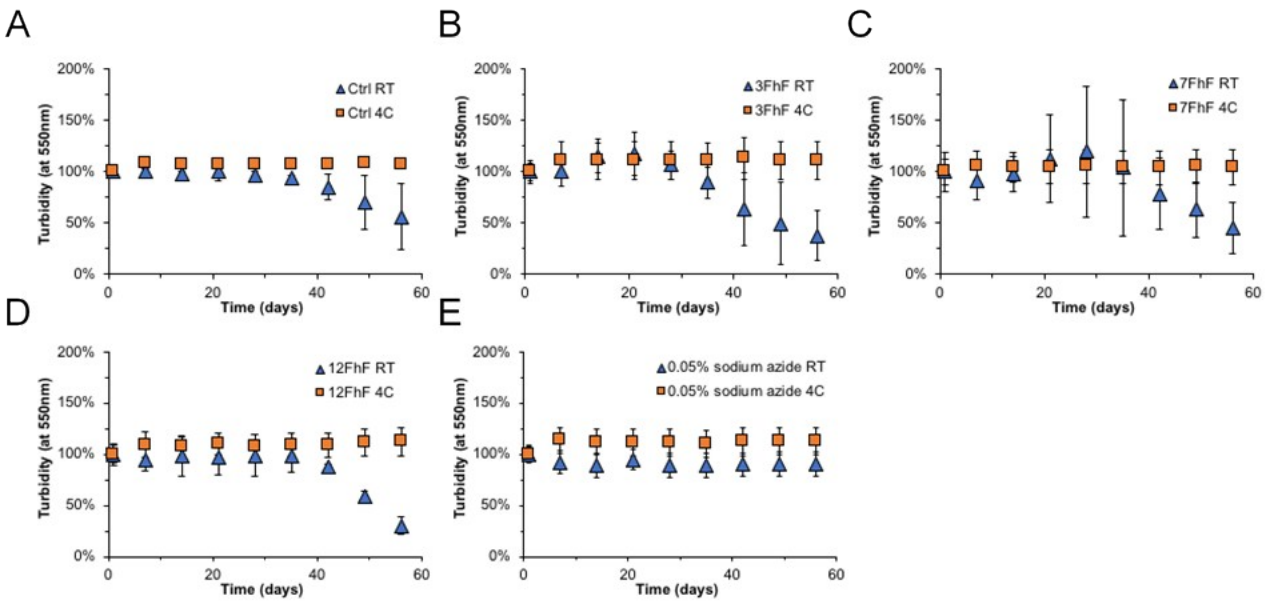
Suppl. Fig. 9: Fluorescence tracing curves of varying plasminogen concentration at fixed [tPA] for digesting physiologically relevant 12FhF annular clots at 37 °C.



Suppl. Fig. 10: Fluorescence tracing curves of varying [tPA] at fixed plasminogen concentration for digesting physiologically relevant 12FhF annular clots at 37 °C.



Suppl. Fig. 11: Comparing digestion rates (initial velocity, abs/min) at 405 nm by S2251 assay at varying [tPA] (0, 200, 500, 1000 ng/mL) with or without 3 mg/mL fibrinogen at 37 °C.



Suppl. Fig. 12: FITC-fibrin structural stability by clot turbidity (at 550 nm) over 8 weeks. Turbidity values were normalized by starting turbidity values of each sample. (A) Unmodified and (B, C, D) physiologically relevant 3, 7, and 12 FhF FITC-fibrin clots were formed in 96 well plates and stored at both RT and 4 °C to determine the long-term stability of the formed clots. Clot structures were monitored every week over 56 days through clot turbidity reads at 550 nm. Turbidity values tracked similarly across all groups. At RT, turbidity values started to decrease after 35 days and reached 30-50% of their starting turbidity at the end of the tested period. While 4°C turbidity values showed no overall changes ($P < 0.05$) for 3, 7, 12 Physiologically relevant (PR) FhF clots and no more than 8% overall changes ($P < 0.05$) for control clots throughout indicating a stable clot structure in all groups over 56-days. As there were no stabilizer agents specifically added to these samples, it is likely that the reduced turbidity over time observed at RT is due to bacterial growth. To test this, an additional untagged fibrin clot was stored in (E) 0.05% sodium azide and no reduction in turbidity was observed over 56-days. Long-lasting stability of a tagged clot substrate greatly expands its utility as it does not need to be formed directly prior to use.