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Thermal shift assay to probe melting of thrombin, fibrinogen, fibrin monomer, and fibrin: Gly-Pro-Arg-Pro induces a fibrin monomer-like state in fibrinogen.

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Structured Abstract

Background—Thrombin activates fibrinogen and binds the fibrin E-domain (K_d ~ 2.8 μM) and the splice variant γ'-domain (K_d ~ 0.1 μM). We investigated if the loading of D-Phe-Pro-Arg-chloromethylketone inhibited thrombin (PPACK-thrombin) onto fibrin could enhance fibrin stability.

Methods—A 384-well plate thermal shift assay (TSA) with SYPRO-orange provided melting temperatures (T_m) of thrombin, PPACK-thrombin, fibrinogen, fibrin monomer, and fibrin.

Results—Large increases in T_m indicated that calcium led to protein stabilization (0 vs. 2 mM Ca^{2+}) for fibrinogen (54.0 vs. 62.3 °C) and fibrin (62.3 vs. 72.2 °C). Additionally, active site inhibition with PPACK dramatically increased the T_m of thrombin (58.3 vs. 78.3 °C). Treatment of fibrinogen with fibrin polymerization inhibitor GPRP increased fibrinogen stability by ΔT_m = 9.3 °C, similar to the ΔT_m when fibrinogen was converted to fibrin monomer (ΔT_m = 8.8 °C) or to fibrin (ΔT_m = 10.4 °C). Addition of PPACK-thrombin at high 5:1 molar ratio to fibrin(ogen) had little effect on fibrin(ogen) T_m values, indicating that thrombin binding does not detectably stabilize fibrin via a putative bivalent E-domain to γ'-domain interaction.

Conclusions—TSA was a sensitive assay of protein stability and detected: (1) the effects of calcium-stabilization, (2) thrombin active site labeling, (3) fibrinogen conversion to fibrin, and (4) GPRP induced changes in fibrinogen stability being essentially equivalent to that of fibrin monomer or polymerized fibrin.

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J.C. Methodology; Investigation; formal analysis; Writing – original draft

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

General Significance—The low volume, high throughput assay has potential for use in understanding interactions with rare or mutant fibrin(ogen) variants.

Keywords

thrombosis; fibrinogen; bleeding; fibrin; thrombin

1. Introduction

Fibrinogen (340 kDa) plays a crucial role in platelet aggregation and fibrin stabilization of a clot. Fibrinogen is a dimer of three separate chains (Aα, Bβ, and γ) that structurally form the central E domain surrounded by two D domains. As a result of alternative splicing, approximately 7% of fibrinogen contains a 20 amino acid extension on the γ chain known as γ' fibrinogen (De Willige et al., 2009). Thrombin cleaves fibrinopeptides A and B from the Aα and Bβ chains, forming fibrin monomers. Fibrinopeptide cleavage exposes the A and B knobs and leads to protofibril extension of the monomers via interactions with complementary holes in fibrinogen or fibrin monomer subunits (Mosesson, 2005).

Thrombin interacts with fibrin via two different binding sites. The low affinity binding (Kd = 1.3 – 2.6) (Meh, Siebenlist, & Mosesson, 1996; Pospisil, Stafford, Fredenburgh, & Weitz, 2003) occurs between thrombin exosite 1 and the E domain of fibrinogen. The high affinity interaction (Kd = 0.11 – 0.25) (Meh et al., 1996; Pospisil et al., 2003) involves thrombin exosite 2 and the γ' chain of fibrinogen, potentially also including a bivalent binding interaction between exosite 1 and the E domain (Lovely et al., 2003; Pospisil et al., 2003). Thrombin can interact with these sites individually or in a bivalent combination (Fredenburgh et al., 2008). Recent papers have shown evidence that high concentrations of thrombin remain bound to the fibrin clot for extended periods of time under flow (Zhu et al., 2016), potentially due to bivalent binding or physical trapping of thrombin within fibrin fibers (Haynes et al., 2017; Kelley & Leiderman, 2019; Zhu et al., 2016, 2018).

In this study, we investigated how various mechanisms during the formation of fibrin might affect the structural stability of fibrin(ogen) monomers and their assemblies. The fibrin polymerization inhibitor GPRP is a synthetic peptide that binds the complementary holes in fibrinogen by mimicking the A knob of fibrinogen (Laudano & Doolittle, 1978; Litvinov et al., 2005). When present at large concentrations, GPRP blocks the protofibril extension of fibrin monomers by binding to the complementary holes in the D domains of fibrinogen (Kononova et al., 2013).

We hypothesized that the exosite interactions between thrombin and fibrin(ogen) have the potential to increase the structural stability of fibrin. D-Phe-Pro-Arg-chloromethylketone (PPACK) is a modified peptide that covalently reacts with the active site serine of thrombin, preventing the cleavage of fibrinopeptides and the formation of fibrin. It has been shown that PPACK does not interact with the exosites of thrombin, which would still allow the high and low affinity exosite interactions (Kaminski & McDonagh, 1987; Meh et al., 1996). PPACK-inhibited thrombin was used in assays of fibrinogen and fibrin to determine the effect of thrombin on the stability of fibin(ogen).

The thermal denaturation temperature (T_m) is typically used as a metric of thermal stability of proteins or protein-ligand complexes. Increased melting temperature in the presence of a binding agent indicates that the bound complex has more stability than the purified protein (Brandts & Lin, 1990; Niesen et al., 2007). Differential scanning calorimetry (DSC) has traditionally been used to determine protein stability by measuring the molar heat capacity of a sample and comparing it to a reference. The DSC peaks are analyzed to determine the denaturation temperature of the sample (Sturtevant, 1987). Although DSC is a powerful method of determining thermodynamic parameters, it is a complex and time consuming approach to determine the denaturation temperature (Durowoju et al., 2017), often requiring large amounts of protein. DSC was used previously to infer structural properties of fibrin(ogen), as well as the effect of the calcium ion on its thermal stability (J. W. Donovan & Mihalyi, 1974; John W. Donovan & Mihalyi, 1985).

The thermal shift assays (TSA) deploys an environmentally sensitive fluorescent dye in order to determine the melting temperature of a protein (T_m). As a protein denatures and unfolds, the dye binds the exposed hydrophobic residues with concomitant increase in fluorescent signal. The melting temperature is the critical point of the fluorescent reading with increasing temperature, determined through the first derivative of the measured fluorescent reading (Huynh & Partch, 2016). By using 384-well plate qPCR equipment for creating linear temperature ramps with time, thermal shift assays are a high throughput and cost effective way to study protein stability (Lo et al., 2004), alone or in the presence of ligands. In this study, we confirmed previous DCS analysis of fibrin(ogen) with thermal shift assays and looked at thermal stability of fibrin monomers in comparison to GPRP-bound fibrinogen. We also investigated the effect of PPACK-thrombin as a fibrin stabilizing agent.

2. Methods

2.1 Materials

The following reagents were obtained and stored as per the manufacturers' instructions: Human Fibrinogen Plasminogen Depleted (FIB1, Enzyme Research Laboratories, South Bend, IN), Human Alpha Thrombin (Haematologic Technologies, Essex Junction, VT), Human D-Phe-Pro-Arg-chloromethylketone-inhibited thrombin (PPACK-thrombin) (Haematologic Technologies, Essex Junction, VT), Gly-Pro-Arg-Pro (GPRP, Millipore Sigma, Burlington, MA), SYPRO Orange Protein Gel Stain (450 nm EX/568 nm EM) (Sigma-Aldrich, St. Louis, MO). γA/γ' fibrinogen (γ' fibrinogen) was generously donated by Dr. John Weisel from the University of Pennsylvania Department of Cell and Developmental Biology.

2.2 Melt Curve Generation

All melt curves were performed using a 384-well plate Roche LightCycler 480. Various mixtures of reagents and buffer conditions were prepared in a final volume of 9 μL with 5X SYPRO Orange and equilibrated for 20 min at 30 C prior to programmed heating. Each well was fluorescently imaged during a linear temperature ramp from 30 C to 90 C (Fig. 1A) over 30 min. First derivative calculations were performed using GraphPad Prism 8 software (Fig.

1B–C). Melt temperature calculations for all samples were determined using LightCycler Thermal Shift Analysis software.

3. Methods

3.1 Fibrinogen and fibrin Melt Curve

Calcium ion is known to stabilize fibrinogen and fibrin (Purves et al., 1978; Weisel, 2005), thus providing a useful validation test for TSA. Fibrinogen T_m was determined in the presence and absence of 2 mM Ca^{2+} (Fig. 2A–B) where a large increase in fibrinogen T_m was detected in the presence of Ca^{2+}. This was further observed in a TSA of increasing concentrations of Ca^{2+} from 0.1 mM to 10 mM with 1 mg/mL fibrinogen (Supplemental Fig. 1). The T_m increased with increasing concentration of calcium ion (Supplemental Table 1). In a similar test of fibrin stability with or without Ca^{2+}, 50 nM thrombin (0.18 μg/mL or ~5 U/mL) was added to 2.9 μM fibrinogen (1 mg/mL) and incubated for 20 min to allow fibrin generation prior to TSA assay, noting that thrombin does not require calcium for activity. As seen with fibrinogen, a large increase in fibrin T_m was detected in the presence of Ca^{2+}, as expected (Table 1). The T_m values of both fibrinogen and fibrin were similar to the thermal denaturation temperatures previously determined by calorimetry (J. W. Donovan & Mihalyi, 1974). The presence of 2 mM Ca^{2+} in the assay shifted the T_m values by approximately 12°C for fibrinogen and 10°C for fibrin. Increases in stability were previously reported by Donovan et. al of 6°C for fibrinogen and 12–13°C for fibrin using differential scanning calorimetry. (John W. Donovan & Mihalyi, 1985). All following experiments were conducted with 2 mM Ca^{2+}. In Fig. 2B, the concentration of active thrombin was much lower than that of the polymerized fibrin (50 nM thrombin vs. 2.9 μM fibrin) so as to have no detectable denaturation signal on its own in the fibrin TSA assay, other than to polymerize the fibrin. In a separate control experiment, the T_m values of thrombin and PPACK-inhibited thrombin (no fibrinogen present) were measured (Fig. 3, raw data included Supplemental Fig. 2). Active site inhibition of thrombin produced a remarkable increase in T_m of 20 °C (Table 1). Fibrin was shown to be fully polymerized with a 20 minute incubation time and 50 nM IIa (Supplemental Fig. 3).

3.2 SYPRO Orange Titration

It has been previously observed that SYPRO orange can interact with a protein and affect the thermal shift assay and the measured Tm (Layton & Hellinga, 2010), though this effect has been found to be minimal at low concentrations of SYPRO less than 10x (Layton & Hellinga, 2011). It was assumed that any effect of SYPRO on the thermal stability would be the same between samples when measuring a shift in Tm with added ligand. To further ensure there was no effect by SYPRO orange dye on the Tm determination of the assays reported, we performed thermal shift assays with varying concentrations of SYPRO between 1x and 10x for fibrinogen, thrombin, and PPACK-thrombin (Supplemental Figs. 4, 5, 6; Supplemental Tables 2, 3, 4, respectively). There was no significant effect of SYPRO concentration on the Tm measured, only an increase in the maximum fluorescence of SYPRO was observed with increasing SYPRO.

3.3 Fibrinogen/fibrin and Gly-Pro-Arg-Pro

The addition of 5 mM GPRP resulted in a 9.3°C increase in the T_m of fibrinogen, clearly demonstrating an increased stability change in fibrinogen due to GPRP occupancy, even in the absence of fibrinopeptide release. Fig. 4A shows a distinct peak for fibrinogen at 61°C. When 5 mM GPRP was present in the assay, the peak shifted to 70°C ($p < 0.0001$). The Tm ~ 70 C of fibrin monomer, produced with GPRP present during thrombin treatment of fibrinogen, was very similar to that of fibrin (no GPPR present) (Fig. 4B). Fibrin was only slightly more stable (~2 C) than fibrin monomer in TSA ($p < 0.0001$). Cleavage of fibrinopeptides by thrombin caused a substantial change in protein stability compared to fibrinogen. Interestingly, the stability of the fibrin monomer was not significantly different from GPRP-fibrinogen as their melting peaks were very similar (Fig. 4). Relative to fibrinogen, statistically significant increases between ΔT_m values of GPRP-treated fibrinogen, fibrin monomer, and fibrin were observed ($p < 0.0001$; N = 3 plates, n = 9 wells). Also, the average ΔT_m values of fibrin (no GPRP) and fibrin monomers (5 mM GPRP) relative to fibrinogen were significantly different (*$p < 0.0001$) (Table 2).

3.4 Effect of high dose PPACK-thrombin on fibrin stability

Clots formed under flow can bind substantial amounts of thrombin due to the antithrombin-I activity of fibrin (Chen & Diamond, 2019; Zhu et al., 2016, 2018) and the continual penetration of fresh prothrombin substrate into clots under flow. Active thrombin bound within the clot through bivalent interactions of thrombin exosite I and II (Kelley & Leiderman, 2019) may have the potential to contribute to clot stability (Meh et al., 1996). Therefore, we tested whether high concentrations of PPACK-thrombin could alter the T_m of fibrin. Fibrin clots were polymerized with active thrombin in the presence of increasing doses of PPACK-thrombin. Unlike the clear shift in the melting temperature between fibrinogen and fibrin (50 nM thrombin), there was no significant difference in T_m between fibrinogen and fibrin with added PPACK-thrombin (Fig. 5, Table 3). While the measured melting temperature of PPACK-thrombin (78.3°C from Table 1) was much higher than that of fibrinogen or fibrin, PPACK-thrombin had no detectable effect on the melting temperature profiles at low concentrations of PPACK-thrombin We conclude that a concentration of 600 nM PPACK-thrombin (22 μg/mL) was not high enough concentration to give a noticeable peak in the thermal shift assay. In contrast in Fig. 6, the PPACK-thrombin T_m peak was easily observed at 78°C at a PPACK-thrombin concentration of 0.2 mg/mL (0.54 μM). The maximum peak increased with larger concentrations of PPACK-thrombin, which is typical of purified systems with increasing concentrations of their reagents (Niesen et al., 2007). The most first and most significant T_m peak for both fibrinogen (~61°C) and fibrin (~71°C) did not shift in this assay by addition of high concentrations of PPACK-thrombin. Loading of fibrin with high doses of PPACK-thrombin had no observable effect on fibrin stability.

3.5 Effect of high dose PPACK-thrombin on γ' fibrin(ogen) stability

To understand the effects of the high affinity thrombin exostie 2 interaction and the γ' chain, purified γ' fibrinogen was used to perform a thermal shift analysis of γ' fibrin(ogen) incubated with and without PPACK-thrombin. Table 5 shows the summary of the T_m's determined. γ' fibrinogen had no significant shift in T_m to unfractionated fibrinogen when

compared on the same plate (results not shown). The T_m of γ' fibrin (64.9 ± 1.3°C) was shifted from γ' fibrinogen (T_m 61.7 ± 0.5°C) but did not have the same stability of unfractionated fibrin. The first derivative curve had a wider and less distinct peak (Supplemental Fig. 7), potentially indicating more heterogeneity in the structure of γ' fibrin or weaker knob-hole interactions between fibrin monomers. 300 nM PPACK-thrombin (5:1 molar ratio with fibrinogen) did not appear to have a significant effect on the T_m of γ' fibrin(ogen). There was a separate, small peak at the determined T_m of PPACK-thrombin (78°C), which appears to be a separate melting event.

4. DISCUSSION

The thermal shift assay detected the calcium-dependent enhancement of fibrinogen and fibrin stability. The conversion of fibrinogen to fibrin also resulted in a large increase in T_m. As indicated by a large and similar increase in T_m, GPRP had the ability to force fibrinogen into a structure similar to that of the fibrin monomer in the absence of fibrinopeptide cleavage. The effect of the knob A mimetic interaction of GPRP on the stability of fibrinogen as indicated by the large increase in Tm has been previously noted. (Kononova et al., 2013)

However, PPACK-thrombin had no effect on fibrin T_m. PPACK covalently binds to the active site of thrombin (Bode et al., 1992). Active site inhibition of thrombin with PPACK resulted in a large enhancement of thrombin thermal stability. Previous reports indicate that PPACK-thrombin maintains exosites that are still able to bind to fibrinogen (Kaminski & McDonagh, 1987; Mosesson & Kaminski, 1990). The high affinity interaction was specifically investigated with a TSA of γ' fibrin(ogen) with and without PPACK-thrombin, with no significant stabilizing effect of PPACK-thrombin on γ' fibrin(ogen) observed.

There was not a large stabilization of γ' fibrin, as seen with unfractionated fibrin. It has been shown that purified γ' fibrin has an altered structure and slower fibrinopeptide B release than both unfractionated and pure γA/γA fibrinogen (Cantero et al., 2019; Cooper et al., 2003). The structural changes might parallel the thermal stability of the γ' fibrin, potentially due to the changes in fibrinopeptide release or changes in knob hole dynamics between the γ' fibrin monomers.

Fibrin had a higher shift in T_m from fibrinogen than both GPRP-bound fibrinogen and the fibrin monomer. While the lateral aggregation of the fibers might explain this difference, interactions between fibrinogen and clot-bound thrombin (Kelley & Leiderman, 2019) might also contribute to this increased stability. PPACK-inhibited thrombin did not affect the melting temperature of fibrinogen or fibrin, even in high concentrations (>0.54 μM). This might suggest that the binding interactions between thrombin's exosites and fibrinogen were not significant enough to add to fibrin thermal stability (Mosesson & Kaminski, 1990).

We have presented a method for determining ligand interactions with fibrin(ogen) and key proteins that are involved in coagulation and hemostasis. We assume that the dye is always rapidly equilibrated with the denatured domains of the unfolding protein. Because the T-ramp occurs over 26 minutes with 35 florescent readings per minute, we also assume that the

protein structure is at a pseudo-equilibrium over the short time of a few seconds when the fluorescence is read in each well (even though unfolding is irreversible and there is no equilibrium between native and unfolded protein) (Lo et al., 2004). The slowest time scale in the measurement may actually be thermal equilibration which should be also rapid for the 9 uL volume in each well.

Some limitations that might exist from the presented results could be in the simplistic determination of the Tm of each protein through the maximum of the first derivative. There are methods that achieve a more accurate determination of the Tm such as fitting the curve to the Boltzman sigmoid function by nonlinear regression (Senisterra et al., 2012). This is most beneficial in TSA's where the melt curve generated has an abnormal shape. However, the curves observed in our experiments fit the typical description as described in Fig. 1, and the first derivative determination served as a straightforward and widely used approach to compare assays directly (Gao et al., 2020; Huynh & Partch, 2016).

Though this study has a limited scope of its observations, the assay can be used in future experiments to determine fibrinogen interactions in which a small volume is required due to rarity of reagents. Specifically, the assay has the potential for studying fibrin(ogen) mutants for instability. For example, Fib$\gamma^{390–396A}$, a mutant of fibrinogen that exhibits reduced binding of transglutaminase factor XIII has been useful in the understanding of the relationship between factor XIII and deep vein thrombosis (Aleman et al., 2014). Our assay would allow further study of this relationship in fibrin clots, requiring small volumes of the mutant type to produce high throughput results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

Aleman MM, Byrnes JR, Wang JG, Tran R, Lam WA, Paola J. Di, Mackman N, Degen JL, Flick MJ, & Wolberg AS (2014). Factor XIII activity mediates red blood cell retention in venous thrombi. Journal of Clinical Investigation. 10.1172/JCI75386

Bode W, Turk D, & Karshikov A (1992). The refined 1.9-A X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human a-thrombin: Structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships. Protein Science, 1, 426–471. [PubMed: 1304349]

Brandts JF, & Lin LN (1990). Study of Strong to Ultratight Protein Interactions Using Differential Scanning Calorimetry. Biochemistry, 29(29), 6927–6940. 10.1021/bi00481a024 [PubMed: 2204424]

Cantero M, Rojas H, Anglés-Cano E, & Marchi R (2019). Fibrin γ/γ' influences the secretion of fibrinolytic components and clot structure. BMC Molecular and Cell Biology, 20(1). 10.1186/s12860-019-0233-0

Chen J, & Diamond SL (2019). Reduced model to predict thrombin and fibrin during thrombosis on collagen/tissue factor under venous flow: Roles of γ'-fibrin and factor XIa. PLoS Computational Biology, 15(8). 10.1371/journal.pcbi.1007266

Cooper AV, Standeven KF, & Ariéns RAS (2003). Fibrinogen gamma-chain splice variant γ′ alters fibrin formation and structure. Blood. 10.1182/blood-2002-10-3150

De Willige SU, Standeven KF, Philippou H, & Ariëns RAS (2009). The pleiotropic role of the fibrinogen γ′ chain in hemostasis. In Blood (Vol. 114, Issue 19). 10.1182/blood-2009-05-217968

Donovan JW, & Mihalyi E (1974). Conformation of fibrinogen: calorimetric evidence for a three nodular structure. Proceedings of the National Academy of Sciences of the United States of America, 71(10), 4125–4128. 10.1073/pnas.71.10.4125 [PubMed: 4530289]

Donovan John W., & Mihalyi E (1985). Clotting of Fibrinogen. 1. Scanning Calorimetric Study of the Effect of Calcium. Biochemistry, 24(14), 3434–3443. 10.1021/bi00335a007 [PubMed: 2864079]

Durowoju IB, Bhandal KS, Hu J, Carpick B, & Kirkitadze M (2017). Differential scanning calorimetry — A method for assessing the thermal stability and conformation of protein antigen. Journal of Visualized Experiments, 2017(121), 1–8. 10.3791/55262

Fredenburgh JC, Stafford AR, Leslie BA, & Weitz JI (2008). Bivalent binding to γA/γ′-fibrin engages both exosites of thrombin and protects it from inhibition by the antithrombin-heparin complex. Journal of Biological Chemistry, 283(5). 10.1074/jbc.M707710200

Gao K, Oerlemans R, & Groves MR (2020). Theory and applications of differential scanning fluorimetry in early-stage drug discovery. In Biophysical Reviews. 10.1007/s12551-020-00619-2

Haynes LM, Orfeo T, Mann KG, Everse SJ, & Brummel-Ziedins KE (2017). Probing the Dynamics of Clot-Bound Thrombin at Venous Shear Rates. Biophysical Journal, 112(8), 1634–1644. 10.1016/j.bpj.2017.03.002 [PubMed: 28445754]

Huynh K, & Partch CL (2016). Current Protocols in Protein Science: Analysis of protein stability and ligand interactions by thermal shift assay. Curr Protoc Protein Sci, 1–19. 10.1002/0471140864.ps2809s79.Current

Kaminski M, & McDonagh J (1987). Inhibited thrombins. Interactions with fibrinogen and fibrin. Biochemical Journal, 242(3). 10.1042/bj2420881

Kelley M, & Leiderman K (2019). A Mathematical Model of Bivalent Binding Suggests Physical Trapping of Thrombin within Fibrin Fibers. Biophysical Journal, 117(8). 10.1016/j.bpj.2019.09.003

Kononova O, Litvinov RI, Zhmurov A, Alekseenko A, Cheng CH, Agarwal S, Marx KA, Weisel JW, & Barsegov V (2013). Molecular mechanisms, thermodynamics, and dissociation kinetics of knob-hole interactions in fibrin. Journal of Biological Chemistry, 288(31), 22681–22692. 10.1074/jbc.M113.472365

Laudano AP, & Doolittle RF (1978). Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. Proceedings of the National Academy of Sciences of the United States of America, 75(7), 3085–3089. 10.1073/pnas.75.7.3085 [PubMed: 277910]

Layton CJ, & Hellinga HW (2010). Thermodynamic analysis of ligand-induced changes in protein thermal unfolding applied to high-throughput determination of ligand affinities with extrinsic fluorescent dyes. Biochemistry. 10.1021/bi101414z

Layton CJ, & Hellinga HW (2011). Quantitation of protein-protein interactions by thermal stability shift analysis. Protein Science. 10.1002/pro.674

Litvinov RI, Gorkun OV, Owen SF, Shuman H, & Weisel JW (2005). Polymerization of fibrin: Specificity, strength, and stability of knob-hole interactions studied at the single-molecule level. In Blood (Vol. 106, Issue 9, pp. 2944–2951). 10.1182/blood-2005-05-2039 [PubMed: 15998829]

Lo MC, Aulabaugh A, Jin G, Cowling R, Bard J, Malamas M, & Ellestad G (2004). Evaluation of fluorescence-based thermal shift assays for hit identification in drug discovery. Analytical Biochemistry, 332(1), 153–159. 10.1016/j.ab.2004.04.031 [PubMed: 15301960]

Lovely RS, Moaddel M, & Farrell DH (2003). Fibrinogen γ' chain binds thrombin exosite II. Journal of Thrombosis and Haemostasis, 1(1), 124–131. 10.1046/j.1538-7836.2003.00027.x [PubMed: 12871549]

Meh DA, Siebenlist KR, & Mosesson MW (1996). Identification and characterization of the thrombin binding sites on fibrin. Journal of Biological Chemistry, 271(38), 23121–23125. 10.1074/jbc.271.38.23121

Mosesson MW (2005). Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis, 3(8). 10.1111/j.1538-7836.2005.01365.x

Mosesson MW, & Kaminski M (1990). The acceleratory effect of thrombin on fibrin clot assembly. In Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis (Vol. 1, Issues 4–5, pp. 475–478). 10.1097/00001721-199010000-00020 [PubMed: 2133225]

Niesen FH, Berglund H, & Vedadi M (2007). The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nature Protocols, 2(9), 2212–2221. 10.1038/nprot.2007.321 [PubMed: 17853878]

Pospisil CH, Stafford AR, Fredenburgh JC, & Weitz JI (2003). Evidence that both exosites on thrombin participate in its high affinity interaction with fibrin. Journal of Biological Chemistry, 278(24), 21584–21591. 10.1074/jbc.M300545200

Purves LR, Lindsey GG, Brown G, & Franks J (1978). Stabilization of the plasmin digestion products of fibrinogen and fibrin by calcium ions. Thrombosis Research, 12(3). 10.1016/0049-3848(78)90318-3

Senisterra G, Chau I, & Vedadi M (2012). Thermal denaturation assays in chemical biology. In Assay and Drug Development Technologies. 10.1089/adt.2011.0390

Sturtevant JM (1987). Biochemical applications kinetics. Annual Review of Physical Chemistry, 38(2), 463–488.

Weisel JW (2005). Fibrinogen and fibrin. Advances in Protein Chemistry, 70 10.1016/S0065-3233(05)70008-5

Zhu S, Chen J, & Diamond SL (2018). Establishing the transient mass balance of thrombosis: From tissue factor to thrombin to fibrin under venous flow. Arteriosclerosis, Thrombosis, and Vascular Biology, 38(7), 1528–1536. 10.1161/ATVBAHA.118.310906

Zhu S, Lu Y, Sinno T, & Diamond SL (2016). Dynamics of thrombin generation and flux from clots during whole human blood flow over collagen/tissue factor surfaces. Journal of Biological Chemistry, 291(44). 10.1074/jbc.M116.754671

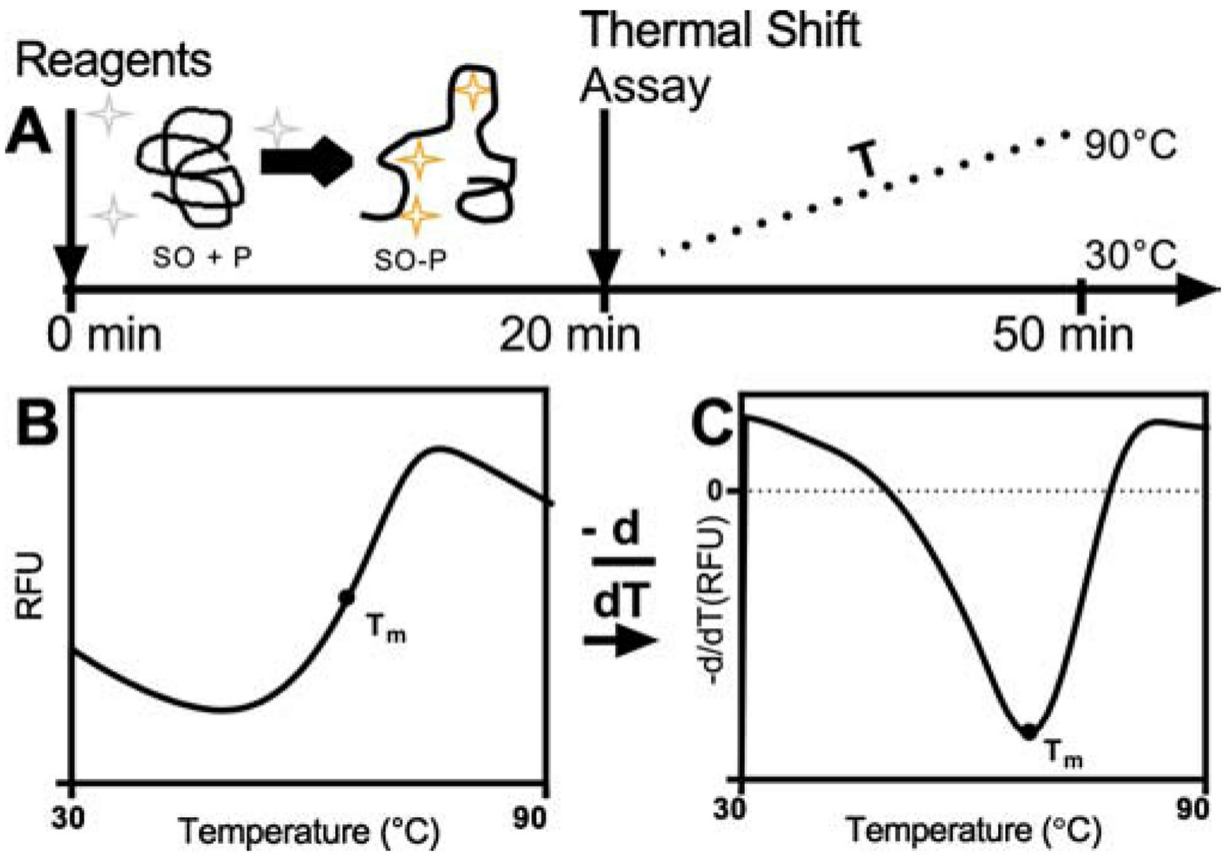

Figure 1.

Graphical Abstract. Thermal shift assay procedure. (A) Reagents were prepared and added to LightCycler 384 well plates. Assay was incubated for 20 minutes to allow for fibrin formation. Reagents then imaged from 30–90°C in LightCycler to develop melt curve. (B) Example melt curve generated from thermal shift assay. T_m is determined by inflection point of curve. (C) Example first derivative curve.

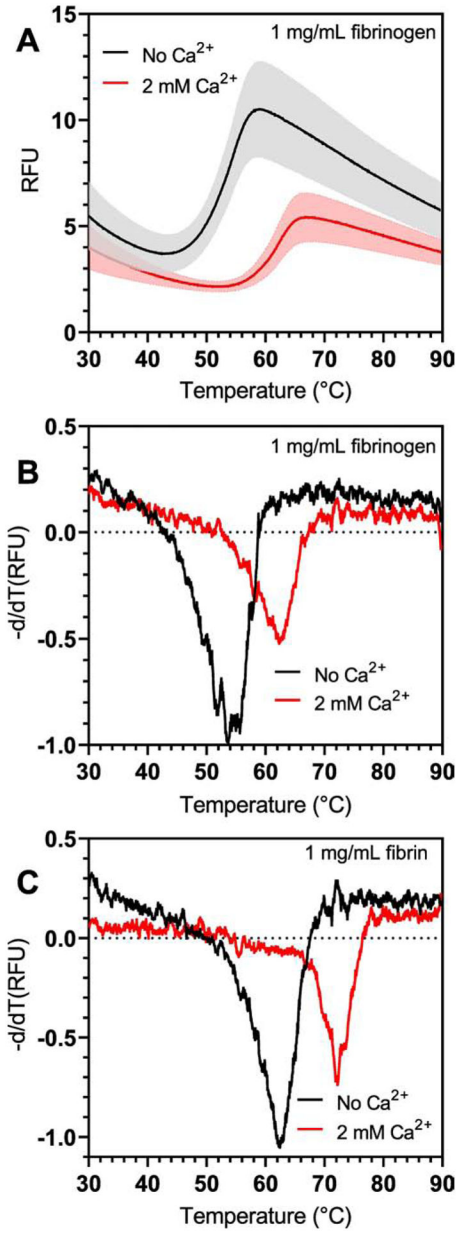

Figure 2.

Generation of melt curves and determination of T_m for fibrinogen and fibrin assays in the presence of Calcium. All assays contained 10x SYPRO. (A) Fibrinogen (1 mg/mL) melt curve. Samples contained either no Ca^{2+} (black) or 2 mM Ca^{2+} (red). (B) First derivative of fibrinogen melt curve. Minimum is T_m of the assay. (C) First derivative of melt curve of fibrin with and without Ca^{2+}. Fibrinogen (1 mg/mL) was incubated with 50 nM thrombin for 20 minutes before performing thermal shift analysis. No Ca^{2+} (black), 2 mM Ca^{2+} (red).

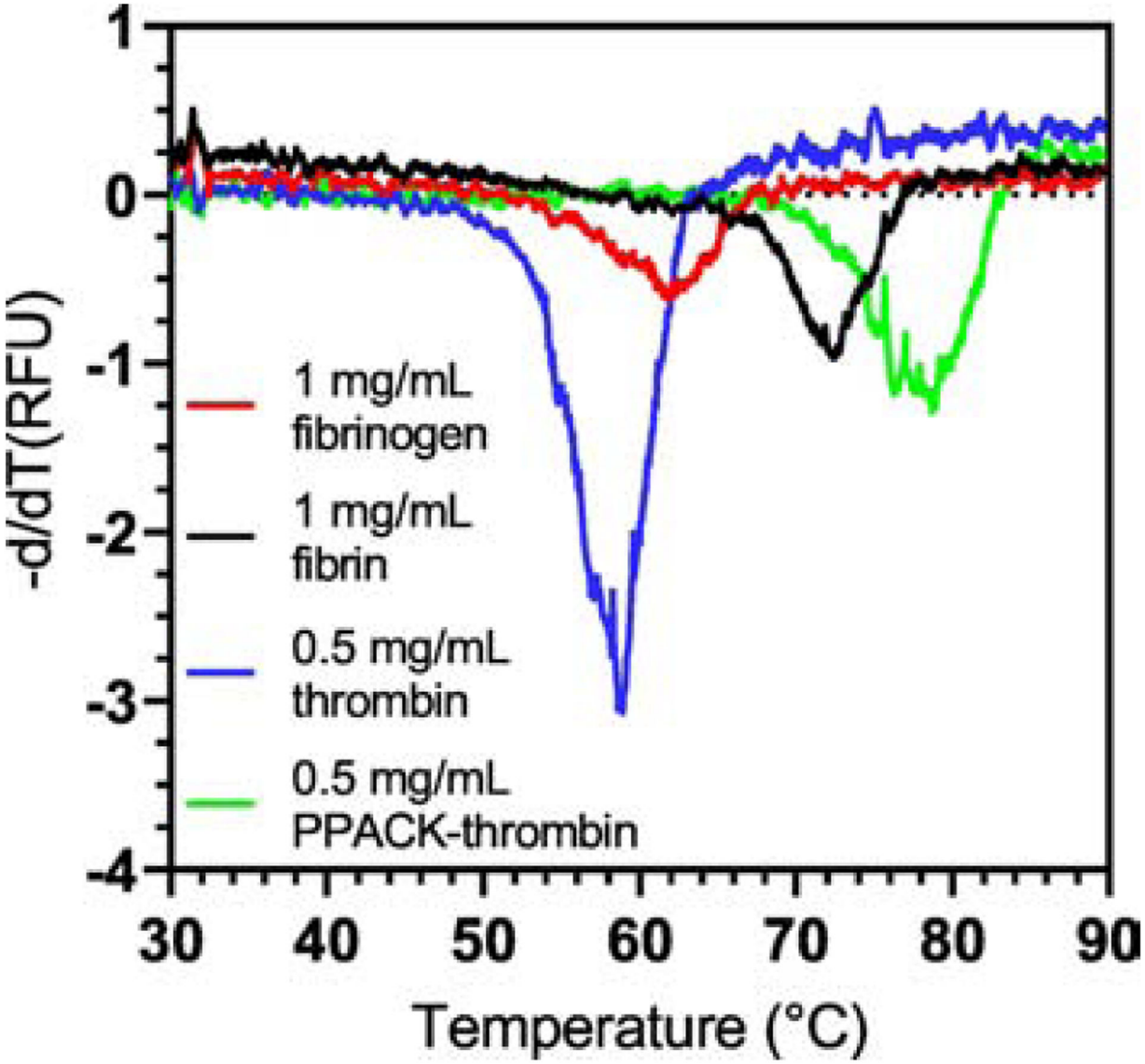

Figure 3.

Representative melting temperature comparison. First derivative of melt curve for PPACK-thrombin (0.5 mg/mL), thrombin (0.5 mg/mL), fibrinogen (1 mg/mL) and fibrin (1 mg/mL fibrinogen; 50 nM thrombin). 2 mM Ca^{2+} and 5x SYPRO. Results are summarized in Table 1.

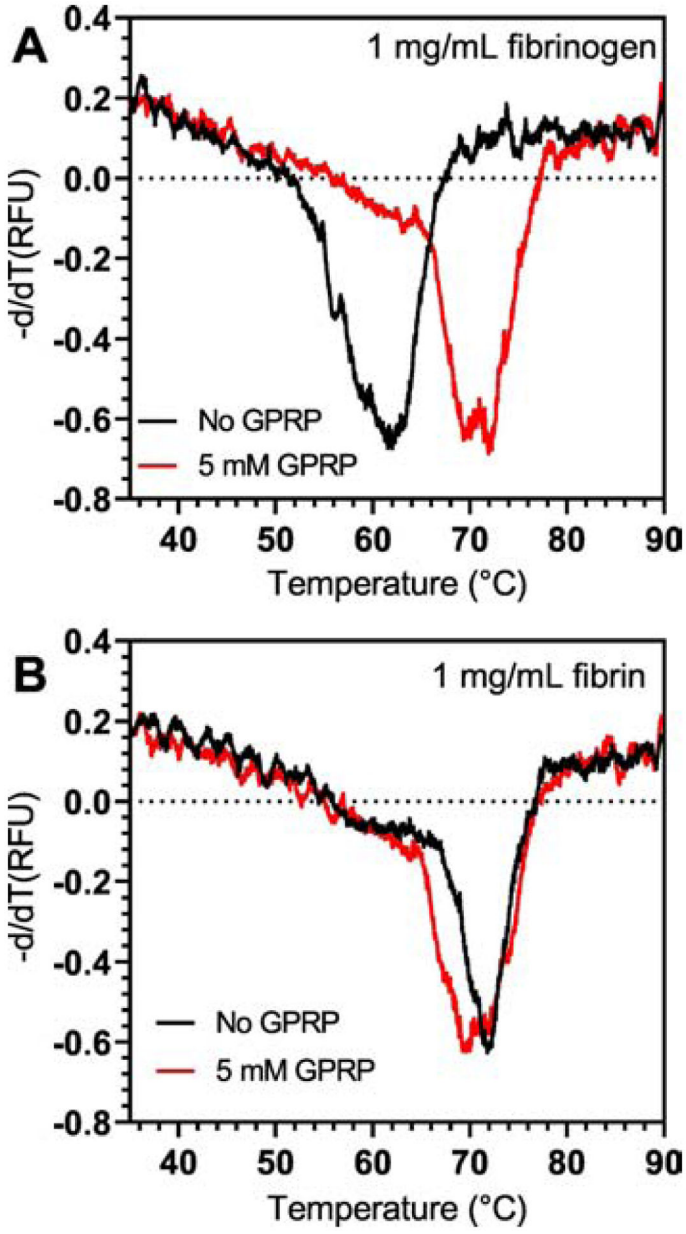

Figure 4.

Representative fibrinogen and fibrin melt curve derivative with and without GPRP. 1 mg/mL fibrinogen, 5x SYPRO, 2 mM Ca^{2+} in each well. (A) Fibrinogen comparison. No GPRP (black) and 5 mM GPRP (red). (B) Fibrin (50 nM thrombin). No GPRP (black) and 5 mM GPRP (red).

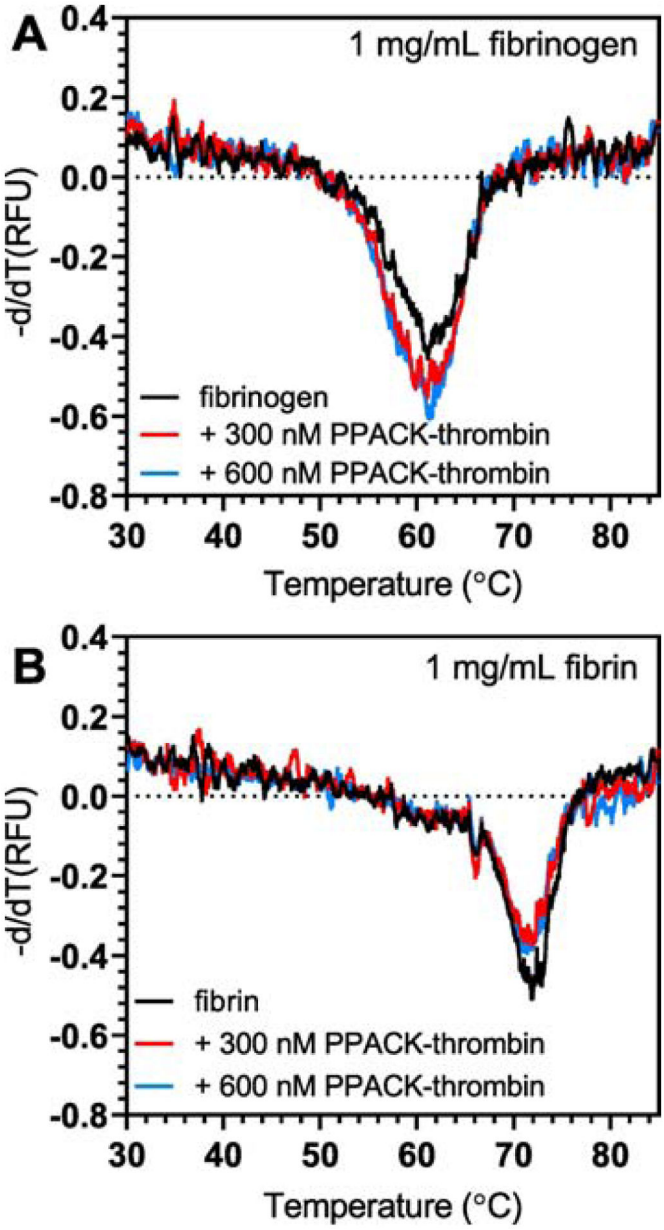

Figure 5.

Graphs of first derivative of melting curve with the addition of PPACK-thrombin. Each well contained 1 mg/mL fibrinogen (3000 nM), 5x SYPRO, and 2 mM Ca^{2+} and was incubated for 20 minutes following the addition of all reagents. (A) Fibrinogen (no thrombin). (B) Fibrin (50 nM thrombin).

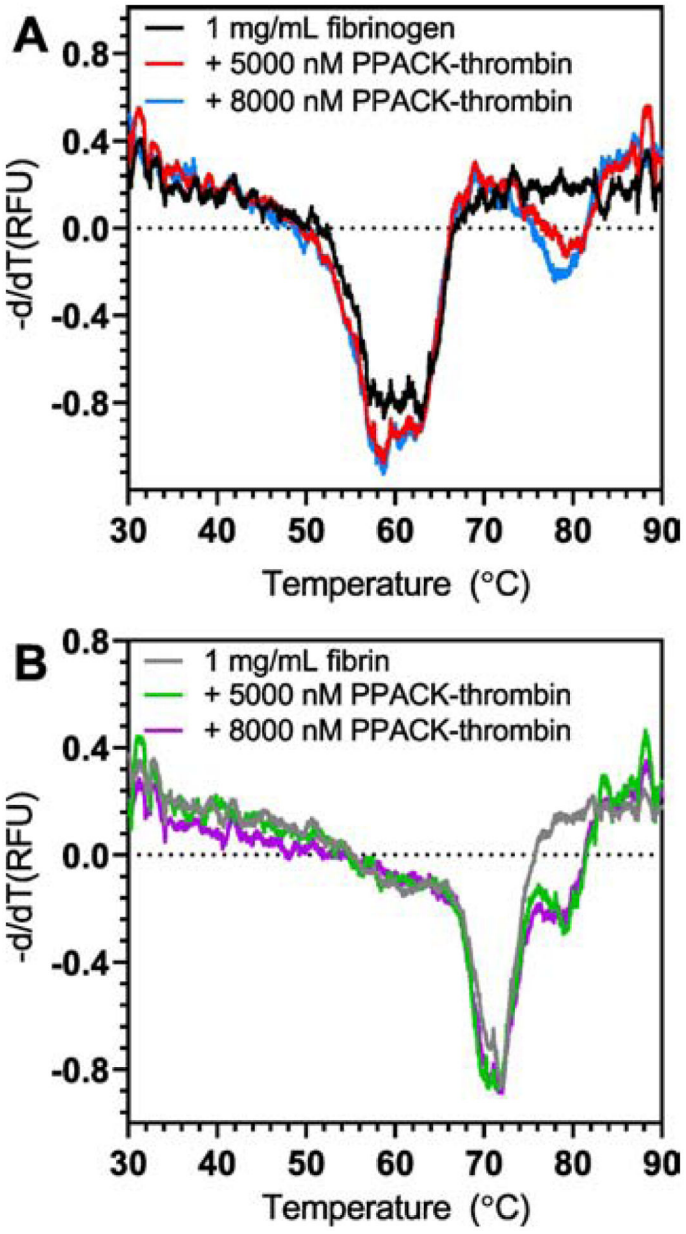

Figure 6.

Representative first derivative of melting curve for fibrinogen and fibrin with added PPACK-thrombin. 1 mg/mL fibrinogen (3000 nM), 5x SYPRO, and 2 mM Ca2+ were used in each well. (A) Fibrinogen with no PPACK-thrombin (black), 5000 nM (0.2 mg/mL) PPACK-thrombin, and 8000 nM (0.3 mg/mL) PPACK-thrombin. (B) Fibrin (70 nM thrombin) with no PPACK-thrombin (black), 5000 nM (0.2 mg/mL) PACK-thrombin, and 8000 nM (0.3 mg/mL) PPACK-thrombin

Table 1

Summary of melting temperatures for pure or reference reagents. Fibrinogen (1 mg/mL), fibrin (1 mg/mL fibrinogen, 50 nM thrombin), thrombin (0.5 mg/mL), and PPACK-thrombin (0.5 mg/mL). 20 minute incubation with all prepared wells and 5x SYPRO before thermal shift assay. n=3 wells for all data.

Sample	Average Tm (°C)
fibrinogen; 0 mM Ca^{2+}	54.0 ± 0.1
fibrinogen; 2 mM Ca^{2+}	62.2 ± 0.3
fibrin; 0 mM Ca^{2+}	62.3 ± 0.3
fibrin; 2 mM Ca^{2+}	72.2 ± 0.3
thrombin	58.3 ± 0.2
PPACK-thrombin	78.3 ± 0.0

Table 2

Summary of shift in T_m measurements from reference fibrinogen. N = 3 plates, n = 9 wells.

Sample	Average ΔTm from ref. fibrinogen (°C)
fibrinogen	0.0 ± 0.4
fibrinogen; 5 mM GPRP	9.3 ± 0.4
fibrin; 5 mM GPRP	8.8 ± 0.4
fibrin	10.4 ± 0.4

Table 3

Summary of shift in T_m measurements from reference fibrinogen on each well plate. N = 1 plates, n = 3 wells

Sample	Average ΔTm from ref. fibrinogen (°C)
fibrinogen	0.0 ± 0.3
fibrinogen; 300 nM PPACK-thrombin	−0.3 ± 0.4
fibrinogen; 600 nM PPACK-thrombin	−0.4 ± 0.3
fibrin	10.1 ± 0.0
fibrin; 300 nM PPACK-thrombin	9.7 ± 0.2
fibrin; 600 nM PPACK-thrombin	9.6 ± 0.3

Table 4

Summary of shift in T_m measurements from reference fibrinogen on each well plate. N = 3 plates, n = 9 wells

Sample	Average ΔTm from ref. fibrinogen (°C)
fibrinogen	0.0 ± 0.3
fibrinogen; 5000 nM PPACK-thrombin	−0.1 ± 0.1
fibrinogen; 8000 nM PPACK-thrombin	−0.2 ± 0.4
fibrin	10.1 ± 0.0
fibrin; 5000 nM PPACK-thrombin	10.1 ± 0.3
fibrin; 8000 nM PPACK-thrombin	10.3 ± 0.5

Table 5

Summary of Tm's measured from γ' fibrin(ogen) with PPACK-thrombin. N=3 plates, n=2 wells. 0.5 mg/mL γ' fibrinogen, 2 mM Ca^{2+}, 10x SYPRO, 25 nM thrombin. 20 minute incubation time.

Sample	Average Tm (°C)
γ' fibrinogen	61.7 ± 0.5
γ' fibrinogen; 300 nM PPACK-thrombin	61.6 ± 0.4
γ' fibrin	64.9 ± 1.3
γ' fibrin; 300 nM PPACK-thrombin	64.6 ± 0.7