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ORIGINAL ARTICLE

Dependence of clot structure and fibrinolysis on apixaban and clotting activator

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Abstract

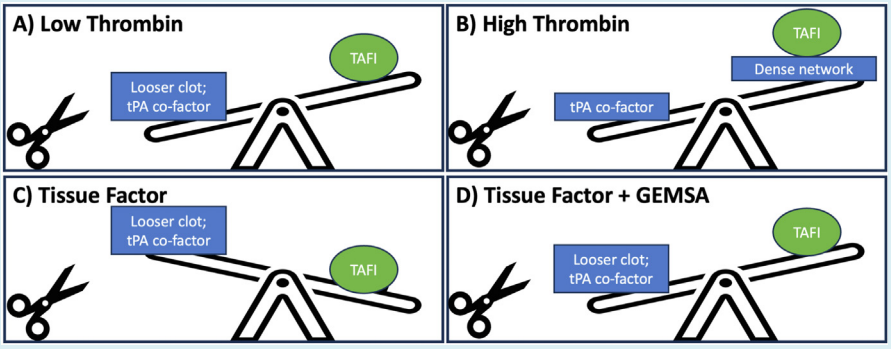
Background: Anticoagulants prevent the formation of potentially fatal blood clots. Apixaban is a direct oral anticoagulant that inhibits factor (F)Xa, thereby impeding the conversion of prothrombin into thrombin and the formation of blood clots. Blood clots are held together by fibrin networks that must be broken down (fibrinolysis) to restore blood flow. Fibrinolysis is initiated when tissue plasminogen activator (tPA) converts plasminogen to plasmin, which binds to and degrades a fibrin fiber. The effects of apixaban on clot structure and lysis have been incompletely studied.

Objectives: We aimed to study apixaban effects on clot structure, kinetics, and fibrinolysis using thrombin (low or high concentration) or tissue factor (TF) to activate clot formation.

Methods: We used a combination of confocal and scanning electron microscopy and turbidity to analyze the structure, formation kinetics, and susceptibility to lysis when plasma was activated with low concentrations of thrombin, high concentrations of thrombin, or TF in the presence or absence of apixaban.

Results: We found that the clotting activator and apixaban differentially modulated clot structure and lytic potential. Low thrombin clots with apixaban lysed quickly due to a loose network and FXa cleavage product's cofactor with tPA; high thrombin clots lysed faster due to FXa cleavage product's cofactor with tPA; TF generated loose clots with restricted lysis due to their activation of thrombin activatable fibrinolytic inhibitor.

Conclusion: Our study elucidates the role of apixaban in fibrinolytic pathways with different clotting activators and can be used for the development of therapeutic strategies using apixaban as a cofactor in fibrinolytic pathways.



KEYWORDS
anticoagulant, apixaban, fibrinolysis, thrombin, thrombosis, tissue factor

Essentials

- Apixaban is an anticoagulant that prevents clot formation.
- We initiated clotting with an anticoagulant while targeting different coagulation pathways.
- Apixaban affected clot structure and lysis differently due to the activator used to initiate clotting.
- Clot degradation was dependent on the balance of clot structure and inhibitors of degradation.

1 | INTRODUCTION

Formation of blood clots is critical to preventing excessive blood loss from tissue injury (eg, from injury, surgery, and ischemia); however, unregulated clotting can lead to complications such as a heart attack, stroke, or venous thromboembolism (VTE). Fibrinogen, a blood plasma protein that is converted to fibrin and polymerizes, provides the structural and mechanical integrity of the clot [1–5]. Eventually, blood clots must be broken down or dissolved, through a process known as fibrinolysis, to restore blood flow. Fibrinolysis is driven by circulating enzymes and zymogens, such as tissue plasminogen activator (tPA) and plasminogen [6]. In settings of increased clotting or impaired fibrinolysis, anticoagulants can be given as therapeutics to prevent clot formation, while tPA can be administered to break down obstructive clots. Careful monitoring of patient status, tPA delivery, and anticoagulant management is required to prevent excess bleeding.

1.1 | Intrinsic vs extrinsic pathway

The coagulation cascade has 2 pathways: 1) the intrinsic pathway—activated by damage inside the vascular system, platelets, damaged endothelium, chemicals, or collagen—and 2) the extrinsic pathway—activated by external trauma that causes blood to escape from the vascular system (Figure 1) [7]. The extrinsic pathway involves tissue factor (TF), which is largely present in endothelial cells and generates thrombin [8]. Activation of clotting with TF requires an additional step to convert prothrombin into thrombin; nonetheless, TF generates large amounts of thrombin leading to quick formation [9]. Both the intrinsic and extrinsic pathways merge at the common pathway upon the formation of factor (F)X. FXa (activated FX) converts prothrombin to its active form, thrombin, an enzyme needed to convert fibrinogen to fibrin. Next, fibrin molecules group into protofibrils to create the matrix of a blood clot. Ultimately, protofibrils form, elongate, and laterally aggregate to form fibrin fibers. This forms the fibrin network [2].

1.2 | Anticoagulants: FXa vs vitamin K antagonists

Anticoagulants, such as FXa inhibitors, prevent blood clot formation [10,11]. Apixaban, a direct FXa inhibitor or direct oral anticoagulant (DOAC), inhibits the conversion of prothrombin to thrombin by competitively binding to the active site of FXa (Figure 1). This prevents the active site from binding prothrombin and FVII, blocking the conversion of prothrombin into thrombin [12]. A recent study identified that anticoagulants, including apixaban, alter clot permeability and lysis in patients [13]. Previous studies show that apixaban increases clot porosity and delays clotting lag time when plasma is activated with TF to form a clot, but no difference was seen when activated with thrombin [14,15]. Experiments with thrombin as the activating factor noticed an enhanced lysis with apixaban [16,17]. Most notably, it was found that lysis is enhanced due to FXa's cleavage products acting as a tPA cofactor [16]. Thus far, lysis with DOACs has only been studied in clots initiated with thrombin, which negates the effect of DOACs and thus might mask its role in clot structure [16].

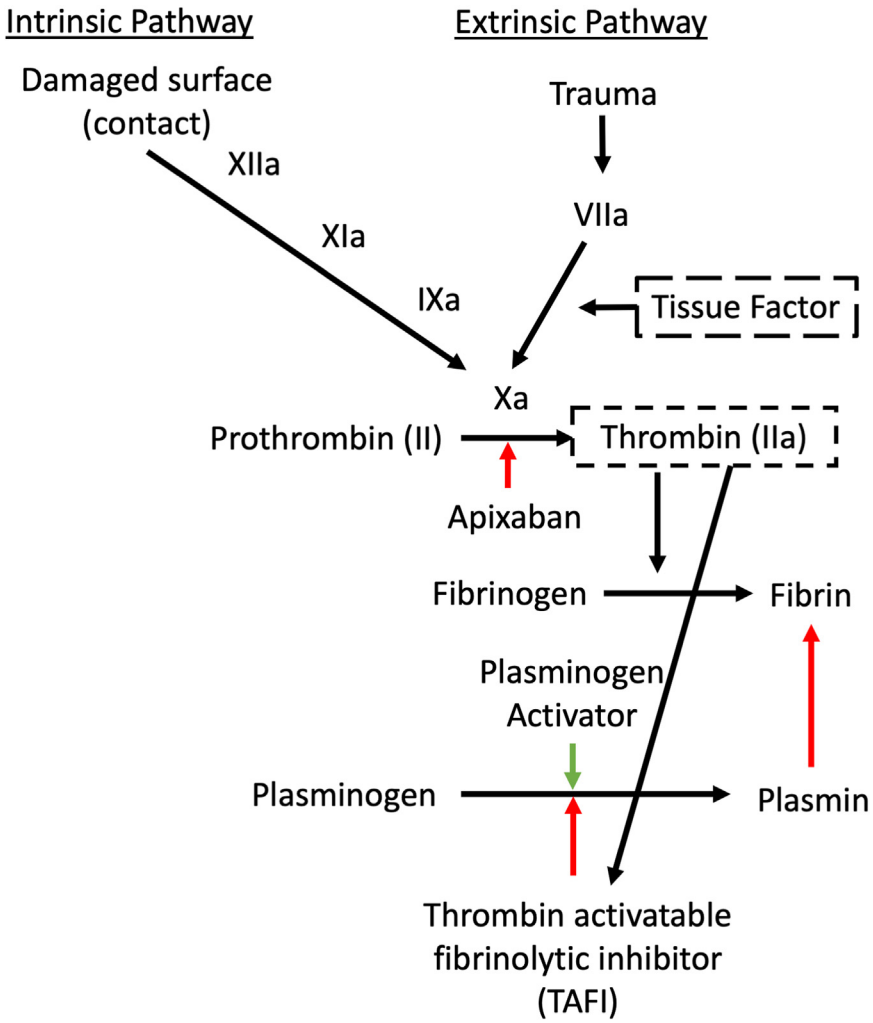


FIGURE 1 Summary of the intrinsic vs extrinsic pathways with select anticoagulants and fibrinolytic inhibitors. Red arrows indicate inhibition. Green arrows indicate activation.

Apixaban can be used as prophylaxis against thrombosis in susceptible patients or as therapy for patients with stroke, VTE, or other thrombotic pathology [18]. Standard preventative doses of apixaban are either 5 mg or 2.5 mg twice a day, depending on the condition for which it is administered [19].

1.3 | Fibrin network structure and fibrinolysis

Fibrin structure is dependent on the composition of plasma proteins, such as fibrinogen or thrombin [20–22], and directly impacts clot lysis [23–28]. Fibrin breakdown, or fibrinolysis, occurs when tPA and plasminogen bind to fibrin fibers. This allows for the cleavage of plasminogen to its active form plasmin. Plasmin proceeds to transversely cleave bonds in fibrin, breaking down the network. Degradation rates differ with various fibrin structures, such as fiber diameter and network porosity by altering the tPA permeability [6]. It is established that individual thin fibrin fibers lyse faster than individually thick fibers while fine fibrin network blood clots, composed of compact thin fibers, lyse slower than coarse blood clots with thicker fibrin fibers and larger pore size [26–28].

1.4 | Fibrinolytic inhibitors

In addition to the standard factors that activate the clotting cascade (ie, thrombin and TF) and initiate lysis (ie, tPA and plasmin), fibrinolytic inhibitors play an important role in regulating hemostasis by stabilizing a clot. Notably, thrombin activatable fibrinolysis inhibitor (TAFI) limits lysis by cleaving the plasmin binding site of fibrin. TAFI can be activated through multiple mechanisms, most commonly thrombin or thrombomodulin [29]. A recent study found that TAFI contributes to the fibrinolytic shutdown in trauma patients [30]. TAFI has also recently been associated with clotting of patients with COVID-19 [31], polycystic ovary syndrome [32], and sickle cell disease [33]. With an increase in the understanding of their role in hemostasis, TAFI inhibitors have been an interesting target for therapeutics [34–36]. In this study, we investigated the role of different clotting activators (low

thrombin, high thrombin, and TF) on apixaban's effects on clot structure, formation kinetics, and lysis potential.

2 | METHODS

2.1 | Platelet-poor plasma

Commercially available pooled human blood plasma (Cone Bioproducts #5781) was warmed to 37 °C prior to all experiments. The buffer used for all experiments was 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (7356-45-9, Sigma-Aldrich) with the addition of 0.1% polyethylene glycol (25322-68-3, Sigma-Aldrich) as previously described [16]. We call this "buffer" for the remainder of the article.

2.2 | Anticoagulant preparation

Apixaban (SML3285-50, Sigma-Aldrich) was resuspended to a stock concentration of 2 mg/mL (0.44 μM) in dimethyl sulfoxide according to the supplier recommendations. Dimethyl sulfoxide does not affect clotting or lysis in our experiments (Supplementary Figure S1). Apixaban was further diluted in buffer for all experiments.

2.3 | Sample preparation for internal anticoagulant/fibrinolytic samples

Samples were prepared by mixing plasma (30% by volume in the final clot: 2.4 mg/mL starting concentration), buffer, apixaban (initial concentration of 200 ng/mL: final concentration of 100 ng/mL), and tPA (final concentration of 5 ng/mL) to achieve the desired concentrations. The concentration 200 ng/mL was chosen for apixaban as it was used in previous studies and has been shown to be the C_{max} with standard dosing [37]. The circulating concentration of tPA is 5 ng/mL [38]. Control samples did not include tPA. Eighty microliters of plasma mix was added to a 96-well plate. Activation mixes were prepared with buffer, $CaCl_2$ (final concentration, 25 mM), and the clotting activator (0.1 U/mL thrombin, 0.5 U/mL thrombin, or 1 pM TF). Thrombin was purchased from Sigma-Aldrich (catalog number T1063), and TF was purchased from Henry Schein (Dade Innovin Reagent, catalog number 10873566). These samples will now be referred to as "Low Thrombin," "High Thrombin," and "TF." Twenty microliters of activator was quickly added using a multichannel pipette to achieve a final clot volume of 100 μL [39].

2.4 | Kinetic tracking of clot formation and lysis

Clot formation was kinetically tracked for 1 hour using a SpectraMax Microplate Reader (Molecular Devices) at 405 nm with 15-second intervals [39–41]. The temperature was set to 37 °C in a humid chamber made up of water wells around the samples. After 1 hour, to retain hydration for the duration of experiments of intrinsic conditions, 100 μL of buffer was overlaid on the corresponding samples. Final concentrations of apixaban and tPA were 100 ng/mL and 5 ng/mL, respectively. Clot lysis was tracked over 10 hours at 405 nm with 2-minute intervals. Experiments were performed in triplicate on 3 separate days.

Additional experiments were performed to inhibit activatable TAFI (TAFIa) using 2-guanidinoethylmercaptosuccinic acid (GEMSA), a known carboxypeptidase B2 inhibitor [42], at a final concentration of 9 mM [43]. Clot formation and lysis assay was performed as described above (final concentrations in fully formed clot of 25 mM $CaCl_2$, 1 pM TF, 200 ng/mL apixaban, and 10 ng/mL tPA). Samples clotted for 1 hour; buffer was added on top to retain hydration.

Parameters for clot formation analyses included lag time formation (time to 5%), maximum optical density (OD), and time to 20%, 50%, 80%, and 95% clot. Lysis parameters included lysis lag time (time from fully formed clot to 5% degradation), time to 95%, 80%, 50%, 20%, and 5% lysis. Rate of formation was recorded as the slope of 20% clot to 80% clot; rate of degradation was recorded as the slope of 80% clot to 20% clot. All analysis on turbidity curves was performed after normalizing data to the first point, relative to the maximum amount.

2.5 | Confocal microscopy

Clots were formed with the same clot conditions as for turbidity with the addition of a fluorescently labeled fibrinogen conjugate of 594 nm (1% by plasma volume, final concentration 4.5 ng/mL). Commercially available pooled human plasma was diluted with buffer to achieve a 30% clot by volume, and $CaCl_2$ (25 mM, final concentration) and thrombin or TF (0.1 U/mL thrombin, 0.5 U/mL thrombin, or 1 pM TF, final concentration) were added immediately prior to addition of the sample to the test well and beginning image collection. Two hundred nanograms per milliliter (final concentration) of apixaban was added for experimental conditions. Images (1024 × 1024 pixels) were captured at 3 different regions of the clot on a Zeiss 78 confocal microscope (n = 3) using a 40× magnification water objective. FIJI (ImageJ) was used to manually measure 27 pore sizes and fiber lengths of every image to track their growth over time. A total of 12 images were analyzed over 2 days. Pore size was defined as the circular region between fibers; the line tool was used to estimate the diameter of this region. The line tool was also used to find the length of an individual fiber [3].

2.6 | Scanning electron microscopy

Clots were formed with conditions as previously described, using lids of 0.5 mL Eppendorf tubes with holes pierced in them with an 18-gauge needle. After 1 hour, fully formed clots were washed with sodium cacodylate (0.2 M, pH 7.4, Electron Microscopy Sciences catalog number # 11652) for 1 hour with a fresh buffer added every 20

minutes to prevent drying. To perform the washes, the centrifuge tube bodies were filled with buffer and attached back to the lids. The holes in the lids allowed for the buffer to flow through the clots by taking advantage of gravity. Clots were fixed overnight in 2% glutaraldehyde. Samples were washed again with sodium cacodylate buffer for 1 hour. Increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) were used to dehydrate the clots. Under a fume hood and on an orbital rocker, the samples were chemically dried with hexamethyldisilazane. The samples were left uncovered overnight (under the fume hood) to completely dry. To mount the samples, carbon tape was placed on specimen stubs, and the dried clots were carefully fastened to the tape. The samples were sputter coated with an 80/20 gold to palladium ratio. Samples were imaged on the Thermo Fisher Scientific Phenom Desktop SEM with 20,000× magnification and 10 kV emission. Diameter and number of fibers per area were measured using FIJI (3 samples from 3 days, 3 images each, 9 total images per condition). The diameter was measured at the intersection of a grid while the number of fibers per area was defined as the fibers across the horizontal line of one of the grid intersections.

2.7 | Protofibril packing

Clots were formed with the same clot conditions as for turbidity: plasma was diluted with buffer to achieve 30% clot; $CaCl_2$ (25 mM, final concentration) and thrombin or TF (0.1 U/mL thrombin, 0.5 U/mL thrombin, or 1pM TF, final concentrations) were added immediately prior to addition of the sample to the test well and beginning image collection. Clot formation was tracked at 405 nm with 15-second intervals at 37 °C. A turbidimetric assay was performed after 1 hour of clotting; readings at a spectrum (300-900 nm at 10 nm intervals) were collected. A custom MATLAB code was written based on previously published equations for the corrected Yeromonahos method [44–46]. This code is accessible at https://github.com/rr901/Protofibril-Packing. Details about the equations can be found in the supplement (Supplementary section 2). The code was used to generate results for protofibril packing and diameter from these measurements.

2.8 | Statistical analysis

All statistical analysis was performed using GraphPad Prism 9.0. Outliers were identified and removed using a Grubbs' test with alpha of 0.05. Normality was confirmed using a D'Agostino and Pearson test for experiments. Two-way analyses of variance were used to compare all turbidimetric parameters in the study among different activators with and without apixaban. Alpha of 0.05 was used for all tests. Rates of formation and degradation were calculated by the linear fit from 20% to 80% clot and 80% to 20% clot, respectively. All experimental data are presented as mean ± SEM; the Supplementary Figures S2–S4 displays individual data points for transparency. Unless otherwise specified, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

3 | RESULTS

3.1 | Role of apixaban in clot structure

Consistent with previous studies, increasing thrombin and TF resulted in denser networks with smaller pores [22] (Figure 2A–C; Supplementary Figure S5). The addition of apixaban differentially modulated the changes in clot structure (Figure 2D–F). Clots generated with a low thrombin concentration and TF with apixaban resulted in a looser network with larger pores and longer fibers compared with their controls (Figure 2G, H). Contrastingly, clots generated with high thrombin did not have a change in pore size or fiber length (Figure 2G, H).

In addition to the microstructural effects of apixaban, changes occurred at the nanoscale level in the protofibril formation. First, we tracked the OD of the clots over time until they were fully formed (Figure 3A–C). Then, we measured the OD of the fully formed clots with a spectrum of wavelengths (Figure 3D–F). Our results were consistent with previous literature that shows a decrease in protofibril packing density for increasing thrombin and TF for control samples (Supplementary Figure S6) [47]. Using the corrected Yeromonahos approach, we calculated that clots generated with low thrombin and apixaban have a looser protofibril packing density per fiber compared with their control, while clots generated with TF and apixaban had denser packing compared with their control (Figure 3G) [44–46]. This differentially impacted the diameter of the fiber—looser packing did not alter the thickness of fibers that had been activated with low thrombin, while denser packing of protofibrils that had been created when plasma was activated with TF resulted in thicker fibers (Figure 3H). Clots with apixaban generated with high thrombin did not alter the protofibril packing density or predicted diameter, which aligned with microscopy (Figure 3G,H).

We next confirmed changes in diameter and density by performing scanning electron microscopy (Figure 4A–F). The results of our control samples were consistent with previous literature that shows a decrease in diameter and increase in lysis with increasing thrombin and TF concentrations (Supplementary Figure S7) [45,47–49]. The diameter of fibers of plasma activated with low thrombin was thinner (without statistical significance) when apixaban was present compared with the control (Figure 4G). The diameter was unaffected when plasma was activated with high concentrations of thrombin; apixaban increased the diameter when plasma was activated with TF (Figure 4G). Density measured by number of fibers per region reflected what was seen with pore size. Apixaban decreased the number of fibers for low thrombin and TF without affecting high thrombin (Figure 4H).

3.2 | Role of apixaban in clot formation and fibrinolysis

The effect of apixaban on formation and lysis was dependent on the activator used to initiate clotting. Apixaban reduced clot formation upon activation with low thrombin, as seen with lower maximum OD, delayed time to 50% clot, and slightly slower rate of formation

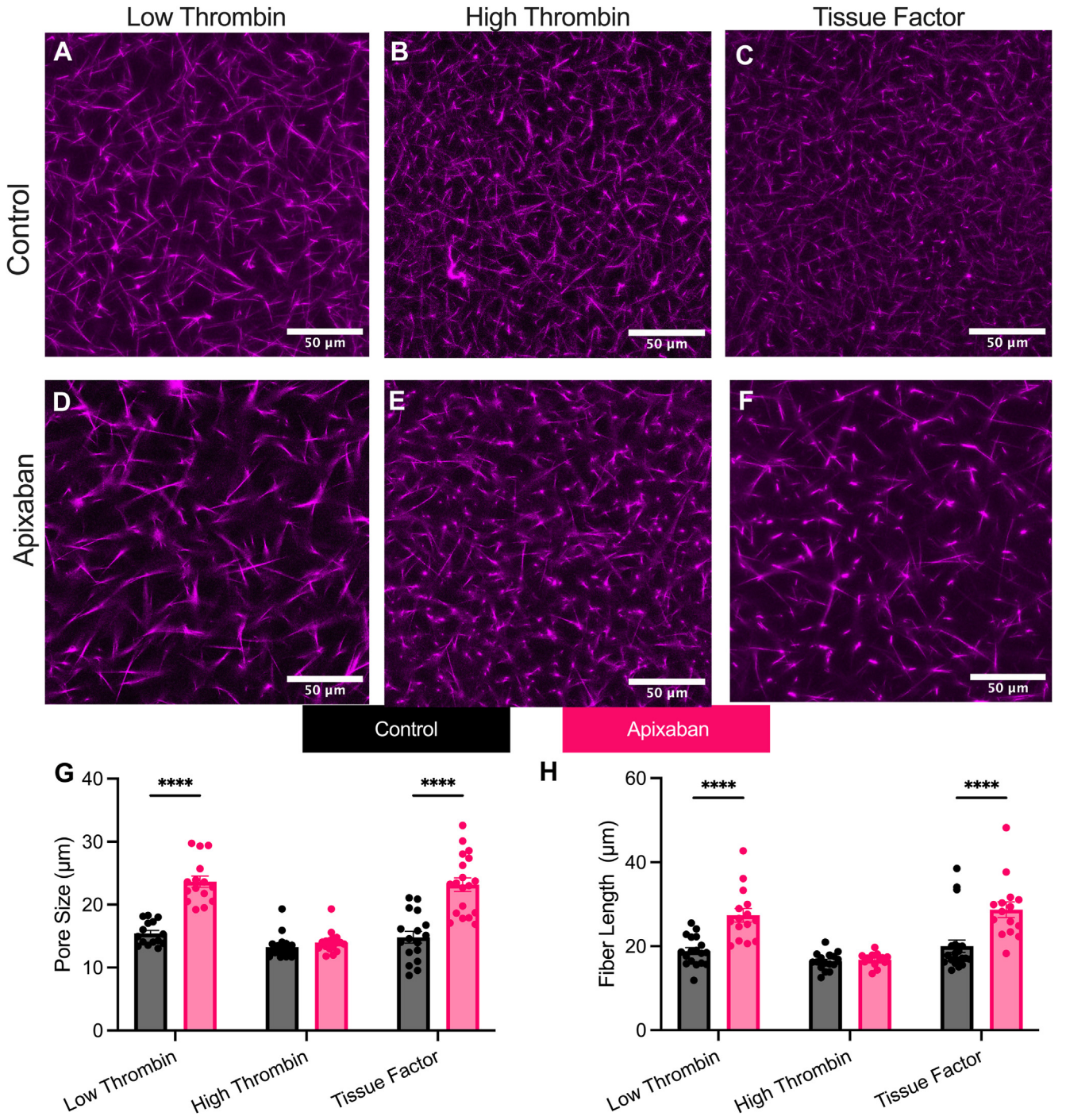


FIGURE 2 Structural analysis of clots with apixaban using confocal microscopy. Confocal microscopy images of clots activated with (A, D) 0.1U/mL (low) thrombin, (B, E) 0.5 U/mL (high) thrombin, and (C, F) 1 pM tissue factor (A–C) without or (D–F) with apixaban. (G) Pore size and (H) fiber length were measured from the images. $N = 18$, 2-way analysis of variance with alpha = 0.05, $^{****}P < .0001$. Scale bar is 50 µm. Data plotted as mean ± SEM.

(Figure 5A, D–F; Supplementary Figure S2). However, apixaban did not impact clot formation when plasma was activated with high thrombin (Figure 5B, D–F; Supplementary Figure S3). The most statistically significant difference on clotting with apixaban compared with the control was seen when the clots were formed with TF activation. With TF, apixaban resulted in a delay in 50% clot formation and a significantly slower rate of formation (Figure 5C, E–F; Supplementary Figure S4). Despite these differences in the timing of clot formation, TF with apixaban did not result in a difference in maximum OD (Figure 5C, D).

Fibrinolysis with internal apixaban was also differentially modulated based on the clotting activator. There was a slight increase in OD after the clot was fully formed due to the addition of buffer to prevent drying [50]. Fiber spreading during the initial lysis can also account for a

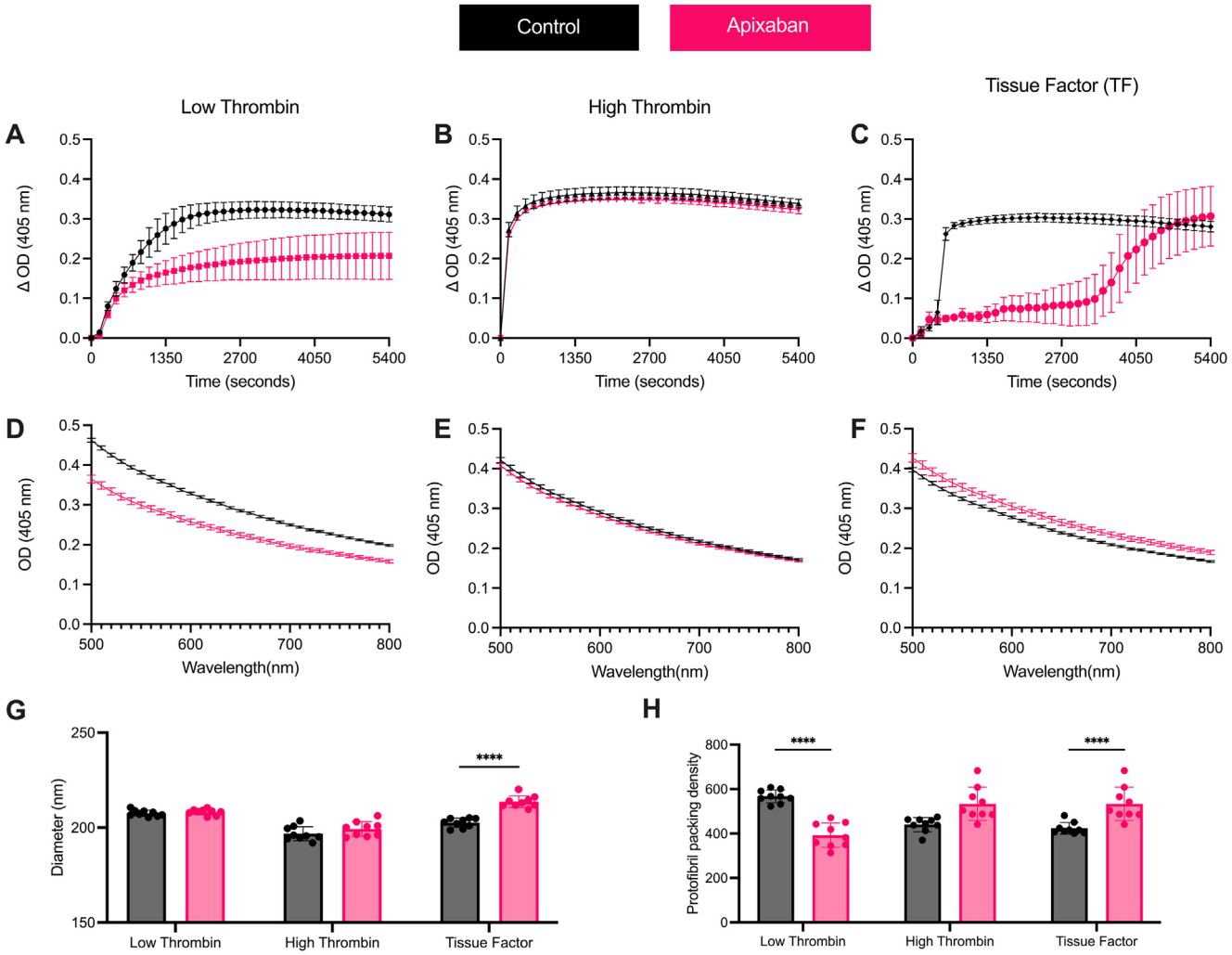


FIGURE 3 Turbidimetric analysis of clot formation and structure. Representative turbidity curves during clot formation with and without apixaban upon activation of plasma with (A) 0.1 U/mL (low) thrombin, (B) 0.5 U/mL (high) thrombin, and (C) 1 pM tissue factor. *N* = 3. (D–F) Turbidimetry curve with respective activators. (G, H) Protofibril packing density and diameter measurements of the respective clots. *N* = 9, 2-way analysis of variance with alpha = 0.05, ****$P < .0001$. OD, optical density.

slight increase in OD. Lysis was accelerated upon activation with low and high thrombin, indicated by the shorter lag time and time to 20% lysis (Figure 5G, H). In contrast, lysis was hindered upon activation with TF, indicated by both a delayed lysis lag time and time to 20% lysis (Figure 5G, H). Lastly, despite the overall faster time to lysis, the rate of degradation was slower upon the addition of apixaban under low thrombin (Figure 5I). In contrast, the rate of degradation was faster when the clots were formed with high thrombin as the activator. Consistent with the delay in lysis for clots when TF was the activator, the rate of degradation was also slower. Trends in lysis due to apixaban were masked with higher tPA concentrations (Supplementary Figure S8).

3.3 | TAFI-dependence on the fibrinolysis potential of clots with apixaban with TF as the activator

We utilized a TAFIa inhibitor, GEMSA, to probe the mechanism of fibrinolytic resistance when clots had been activated with TF and apixaban (Figure 6A). As previously described, clotting was significantly delayed with apixaban (Figure 6B). Initial lysis was slightly faster with apixaban, as evidenced by a slightly earlier time to 5% lysis (Figure 6C). There was an earlier, yet statistically insignificant, time to 50% lysis (Figure 6D) as the degradation of clots with and without apixaban was comparable.

A summary of results can be found in the Table.

4 | DISCUSSION

Anticoagulants are essential in preventing and treating life-threatening blood clots [51,52]. In particular, apixaban is a widely known and prescribed anticoagulant that targets FX, preventing its activation to FXa and the ultimate conversion of prothrombin into thrombin that would subsequently form the fibrin network [37]. The influence of apixaban on the fibrin network structure, if a clot were to

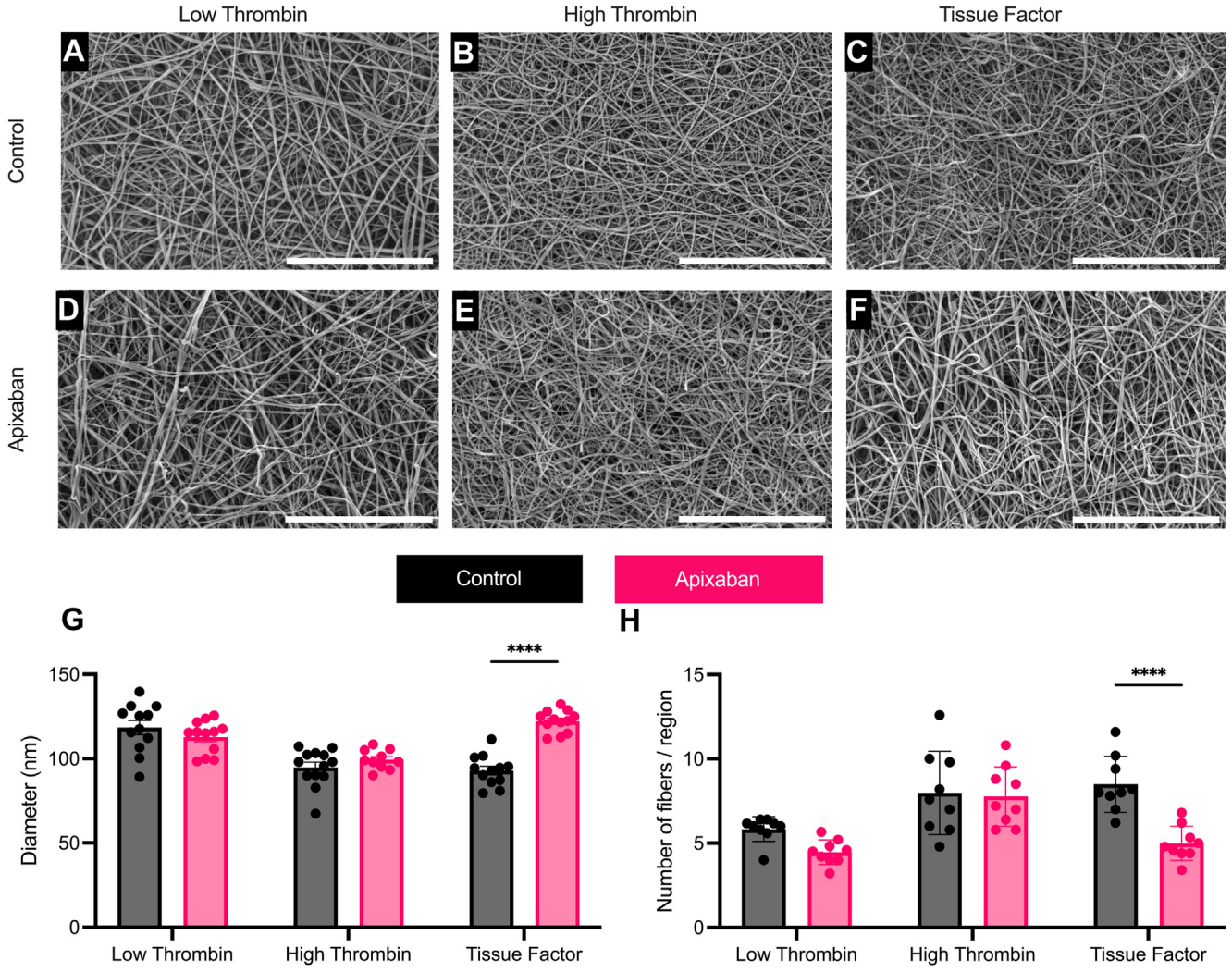


FIGURE 4 Structural analysis of clots with apixaban using scanning electron microscopy. Scanning electron micrographs upon activation with (A, D) 0.1 U/mL (low) thrombin), (B, E) 0.5 U/mL (high) thrombin, and (C, F) 1 pM tissue factor of samples (A–C) without and (D–F) with apixaban. (G) Impact of apixaban and different activators on fibrin fiber diameter. (H) Impact of apixaban and different activators on number on fiber density. Region is 7 μm^2. Scale bar is 50 μm. Data plotted as mean ± SEM. n = 9, 2-way analysis of variance with alpha = 0.05, $^{****}P < .0001$.

form, has been studied previously. Patients undergoing treatment with apixaban were found to have more clot permeability compared with normal pooled plasma [13]. It was seen *in vitro* that clots with apixaban formed with TF as the activator resulted in a looser network while activation with thrombin resulted in no difference in density compared with controls [14]. Another study identified that apixaban sped up fibrinolysis due to a cofactor role; however, this study focused on clots that had been formed with thrombin as the activator [14,16]. Here, we not only studied 2 concentrations of thrombin but also replicated trauma conditions by initiating clot formation with TF. This distinction is particularly important for apixaban as TF is upstream of FX while thrombin is downstream of FX. Furthermore, previous studies only added the anticoagulant as a preventative measure before the clot was formed; in the present study, we did additional experiments that probed the role of secondary prevention, or reactive, apixaban when delivered to a fully formed clot [53].

The current structural studies demonstrated that apixaban differentially modulated the fibrin network pore size, fiber diameter, and protofibril packing when the clots were formed when plasma was activated with low thrombin, high thrombin, and TF (Figures 2–4). The unique network structures are dependent on the clot formation kinetics that dictate the rate at which protofibrils elongate and laterally aggregate to form fibers. We and previous literature show that increasing thrombin results in denser fibrin networks (Supplementary Figure S5) and denser protofibril packing (Supplementary Figure S6) [47]. The low thrombin and TF with apixaban had delayed clotting compared with their control counterparts, which allowed for more protofibril elongation resulting in longer fiber lengths. TF with apixaban's slower rate of formation also resulted in less protofibril packing density and subsequently thicker fibers. Activation with high thrombin did not affect clotting kinetics; therefore, the formation and elongation of protofibrils and thus network structure was comparable with

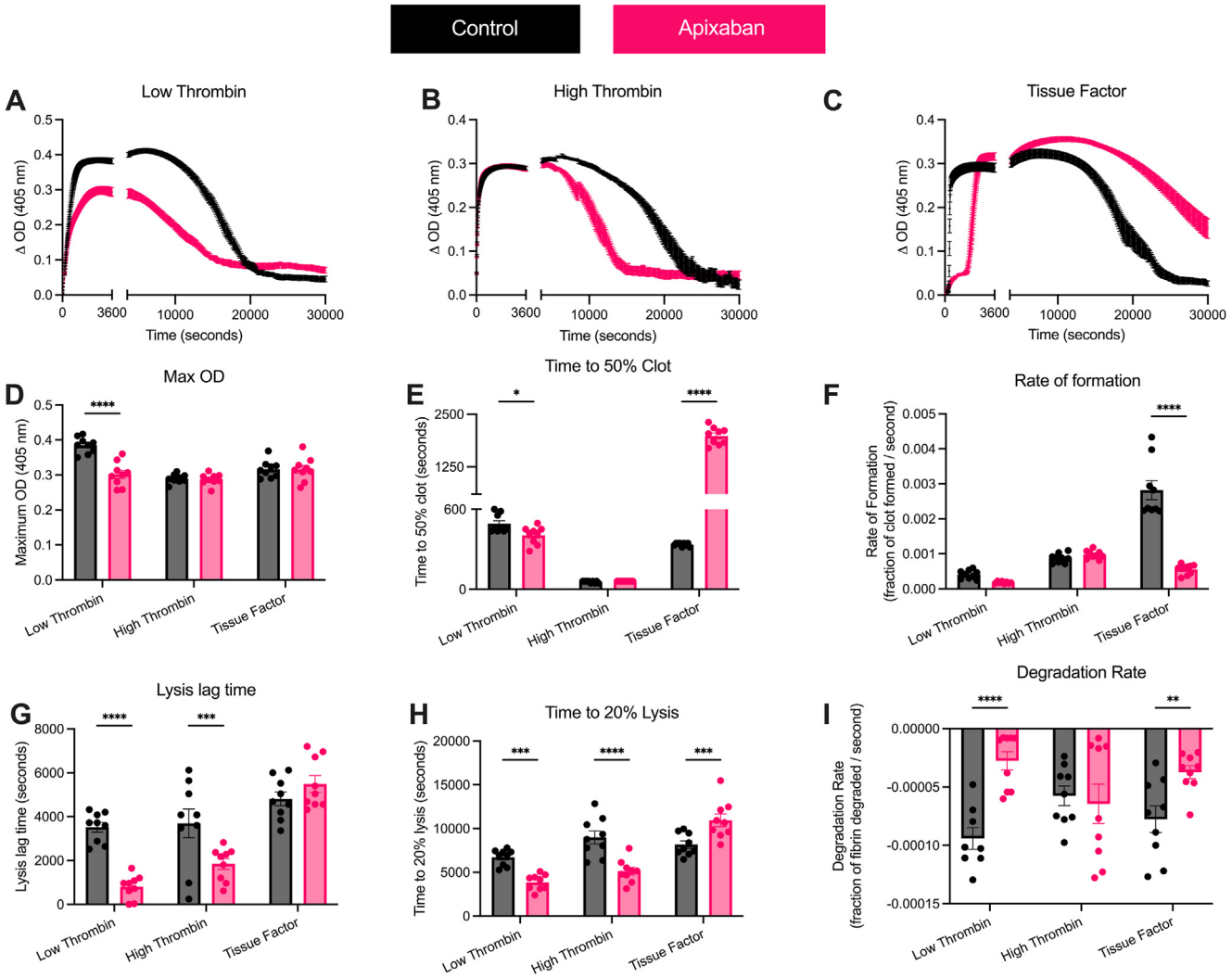


FIGURE 5 Clot formation and lysis kinetics. Clot formation and lysis curves under activation with (A) 0.1 U/mL (low) thrombin, (B) 0.5 U/mL (high) thrombin, and (C) 1 pM TF. Parameters measured include (D) maximum optical density, (E) time to 50% clot, (F) rate of formation, (G) lysis lag time, (H) time to 20% lysis, and (I) degradation rate. $N = 9$, 2-way analysis of variance with alpha = 0.05, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Scale bar is 50 μm. Data plotted as mean ± SEM with individual data points ($n = 9$). OD, optical density.

and without apixaban. It is established that denser fibrin networks lyse slower than looser networks [27,28]; however, here, we noticed that loose networks seen for the TF with apixaban lysed slower than the denser network without apixaban (Figure 5). Lysis was enhanced under both low and high thrombin initiation of clotting when treated with apixaban. The looser fibrin network structure generated from apixaban with plasma activated with low thrombin, as well as FXa cleavage product as a tPA cofactor, aided in the faster lysis. Although activation with high thrombin did not affect fiber or network structure, apixaban sped up lysis compared with the control. We propose that there was no difference in clot formation due to sufficient thrombin present to overcome apixaban's role in inhibiting further conversion of prothrombin to thrombin. Nonetheless, apixaban allowed for the persistence of a large amount of the FXa cleavage product in addition to FXa inactivation to behave as a cofactor with tPA to enhance lysis [16]. Unlike the thrombin scenarios and despite the significantly looser network that apixaban and TF generate, lysis was significantly delayed compared with the control. This difference, once again, points to a potentially different mechanism between the thrombin and TF activation when treated with apixaban. It was previously shown that apixaban with the initiation of clotting with thrombin is independent of TAFI activation [16], but this was not studied for clots formed with TF as the activator. We propose that the large amount of thrombin generated from TF resulted in surplus activation of TAFI, resulting in delayed lysis [54]. We confirmed this mechanism by inhibiting TAFIa and saw enhanced lysis compared with the control. In conjunction with tPA, TAFIa inhibitors could be a useful therapeutic target for patients with thrombosis [55,56].

The role the activator plays in clot structure aids in the understanding of how to treat a patient taking apixaban, depending on the method in which a clot formed. Notably, VTE after trauma and surgery may generate clots from TF while stroke and heart attacks are

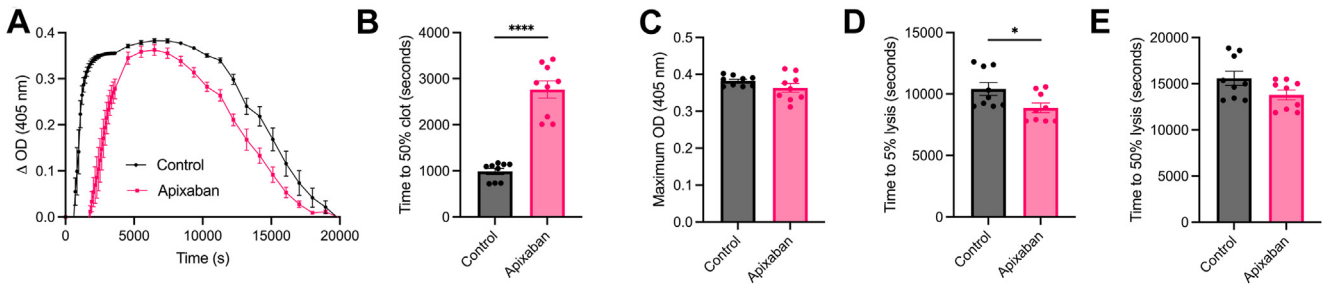


FIGURE 6 Inhibition of activatable thrombin activatable fibrinolysis inhibitor using guanidinoethylmercaptosuccinic acid. (A) Clot formation and lysis with and without apixaban with the addition of guanidinoethylmercaptosuccinic acid in both samples. Time to (B) 50% clot formation, (C) maximum optical density, (D) 5% lysis, and (E) and 50% lysis were recorded. $^{**}P < .01$. Data plotted as mean ± SEM with individual data points (n = 9). OD, optical density.

thrombin-mediated. This innate difference implies that it may be beneficial in trauma settings to add TAFIa inhibitors. In the case of heart attack or stroke, TAFIa inhibitors could result in excess bleeding; in these situations, it could be more helpful to treat with reactive apixaban. This is also an important consideration for persons with hemophilia who have been shown to have impairment of the TAFI pathway [54]. Similarly, patients with deficiency in activated protein C would require particular attention as activated protein C acts dependently on TAFI, resulting in profibrinolytic behavior [57].

The balance of TAFIa inhibitors for treatment would be an important consideration; research has shown that laboratory inhibitors for TAFIa, such as the one used here (GEMSA), potato tuber carboxypeptidase inhibitor [58], or nanobodies [59], have a biphasic effect on lysis. High concentrations speed up lysis, but low concentrations stabilize the clot. Potential drug candidates have to balance the profibrinolytic effect with the risk of bleeding [56]. Our results confirm that the clinical development of TAFIa inhibitors would require methodical dosage and careful monitoring to avoid hemorrhagic stroke or excess bleeding.

In some instances, standard apixaban treatment may be ideal to prevent thrombosis and aid in lysis, which we saw *in vitro* with the clots that formed with thrombin as the activator. For example, cancer patients are at high risk for developing VTE, requiring standard prophylactic treatment; apixaban has shown success in preventing VTE in patients initiating chemotherapy but caused adverse bleeding in more patients as compared with the placebo [60]. Administering a TAFIa inhibitor could amplify this risk. It has been shown that TAFIa aids in thwarting metastasis, specifically in breast cancer [61].

TABLE Summary of clot formation and lysis with and without apixaban using various activators.

Clotting activator condition (apixaban compared with control)	Time to 50% clot	Max OD	Pore size	Fiber diameter	Lysis lag time
Low thrombin	—	↓	↑	—	↓
High thrombin	—	—	—	—	↓
TF	↑	—	↑	↑	↑
TF + GEMSA	↑	—	N/A	N/A	↓

Arrows indicate relationships between clots when clotting was activated with each reagent with addition of apixaban vs. without apixaban present. A dashed line indicates no change.

GEMSA, 2-guanidinoethylmercaptosuccinic acid; N/A, not applicable; OD, optical density; TF, tissue factor.

While we were able to characterize the role of clotting activator and apixaban on fibrin structure and fibrinolytic potential, there are some limitations that could be addressed in the future. First, here we focused on one FXa inhibitor, apixaban, at a single dose, while we could also probe if similar behaviors occur at other dosages or with other FXa inhibitors such as rivaroxaban. Alternatively, we could study direct thrombin inhibitors, such as dabigatran. Furthermore, we targeted the extrinsic and common pathways; we could also study the intrinsic and contact pathways, which would advise about patients with deficiencies in factors such as FXII. The current work was completed under static conditions, which allowed us to fix the concentrations of tPA and apixaban without the need for continuous resupply. Future work could introduce fluid flow to increase the physiological relevance and mimic dynamic *in vivo* conditions. We hypothesize that fluid flow would increase the network density of each clot condition without altering the trends between clotting activator or role of apixaban [62]. Likewise, we suspect that the trends in clot formation and lysis would be consistent with static conditions as the dominating feature driving susceptibility to lysis was FXa–tPA cofactor enhancing lysis vs TAFI restricting lysis. Nonetheless, lysis could be universally dampened if either factor is removed from the system due to flow. Lastly, our study focused on platelet-poor plasma to isolate the role of fibrinogen and clotting activator on apixaban; future directions could systematically incorporate cells, such as platelets and red blood cells, and eventually use whole blood to increase the physiological relevance. We hypothesize that red blood cells would affect the structure of the clot limiting tPA diffusion universally, but the role of the cofactor and TAFI would not be altered. Furthermore, we hypothesize that platelets would exacerbate the restriction of lysis during TF activation with apixaban due to additional activation of TAFI by activated platelets [63]. Our results show promising evidence for the value of TAFIa inhibitors; future research could use mouse models to probe their efficacy and safety. Future

directions could evaluate combinatorial treatment dependent on the mechanism of clot formation.

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AUTHOR CONTRIBUTIONS

R.A.R. and M.S. performed experiments, analyzed data, and wrote the manuscript. V.T. and J.G. oversaw the direction of experiments and analyzed data. All authors edited the final manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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