Structure of shear-induced platelet aggregated clot formed in an in vitro arterial thrombosis model

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Abstract:
The structure of occlusive arterial thrombi is described in this paper. Macroscopic thrombi are made from whole blood in a collagen-coated, large-scale stenosis with high shear flow conditions similar to an atherosclerotic artery. The millimeter-sized thrombi are harvested for histology and scanning electron microscopy. Histological images showed 3 distinctive structures of the thrombus. A) The upstream region showed string-like platelet aggregates growing out from the wall to protrude into the central lumen, while RBCs were trapped between the strings. The strings were >10x as long as they were wide and reached out to join the strings from the opposite wall. B) Near the apex, the platelet strings coalesced into a dense mass with microchannels that effectively occludes the lumen. C) In the expansion region, the thrombus ended abruptly with an annulus of free blood in the flow separation zone. Scanning electron microscopy showed dense clusters of spherical platelets upstream and downstream, with amorphous platelets in the occluded throat consistent with prior activation. The total clot is estimated to contain 1.23 billion platelets with pores on the order of 10-100 microns. The results reveal a complex structure of arterial thrombi that grow from their tips under high shear stress to bridge the 2.5 mm lumen quickly with VWF-platelet strings. The occlusion leaves many microchannels that allow some flow through the bulk of the thrombus. This architecture can create occlusion or hemostasis rapidly with minimal material, yet remain porous for potential delivery of lytic agents to the core of the thrombus.

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Key points

- Shear-induced platelet aggregated clot is platelet and VWF rich and clusters of billions of platelets aggregated to occlude a 2.5mm lumen.
- Platelet aggregates grew as long, thin strings reaching across the lumen, then coalesce sideways, creating unusual structure.

Abstract

The structure of occlusive arterial thrombi is described in this paper. Macroscopic thrombi are made from whole blood in a collagen-coated, large-scale stenosis with high shear flow conditions similar to an atherosclerotic artery. The millimeter-sized thrombi are harvested for histology and scanning electron microscopy.

Histological images showed 3 distinctive structures of the thrombus. A) The upstream region showed string-like platelet aggregates growing out from the wall to protrude into the central lumen, while RBCs were trapped between the strings. The strings were >10x as long as they were wide and reached out to join the strings from the opposite wall. B) Near the apex, the platelet strings coalesced into a dense mass with microchannels that effectively occludes the lumen. C) In the expansion region, the thrombus ended abruptly with an annulus of free blood in the flow separation zone. Scanning electron microscopy showed dense clusters of spherical platelets upstream and downstream, with amorphous platelets in the occluded throat consistent with prior activation. The total clot is estimated to contain 1.23 billion platelets with pores on the order of 10-100 microns.

The results reveal a complex structure of arterial thrombi that grow from their tips under high shear stress to bridge the 2.5 mm lumen quickly with VWF-platelet strings. The occlusion leaves many microchannels that allow some flow through the bulk of the thrombus. This architecture can create occlusion or hemostasis rapidly with minimal material, yet remain porous for potential delivery of lytic agents to the core of the thrombus.
Introduction

Arterial thrombosis in a stenosed coronary artery or carotid artery can lead to a myocardial infarction or an ischemic stroke. The formation of an occlusive thrombus in these arteries blocks blood flow, resulting in the patient's death. To stop blood flow, the thrombus must have formed under a high shear rate of up to 400,000 s\(^{-1}\) \(^1\) and be able to resist arterial blood pressure of over 175 mmHg \(^2\).

A thrombus can be distinguished by its mechanism of formation. A coagulation clot follows the classic Virchow's triad, which consists of stagnant blood flow, endothelial disruption, and hypercoagulability \(^3\). The primary structural component of a coagulation clot is fibrin, which is the final product of the coagulation cascade that entraps large amounts of RBCs. A coagulation clot is often referred to as a “red clot” because it appears to be red in color due to the presence of a large number of RBCs \(^4,5\). However, a coagulation clot can appear to be white in color if the clot is formed from artificially generated platelet-rich plasma (PRP) lacking in RBCs but having an abundance of fibrin. Meanwhile, Casa, et al. \(^6\) proposed an alternative triad for shear-induced platelet aggregation (SIPA) clot formation: (i) a pathologically high shear rate, (ii) a collagen surface for von Willebrand Factor (VWF) attachment, and (iii) platelets and VWF in sufficient concentrations. Thus, a SIPA clot is composed predominantly of platelets and VWFs, with a few RBCs; further supported by Ku and Flannery \(^7\)'s study in which 80\% of a SIPA clot consisted of platelets. SIPA clots are commonly called “white clots” because of the lack of RBCs. A coagulation clot is implicated in deep venous thrombosis; whereas, a SIPA clot is more strongly implicated in arterial thrombosis \(^5,8\). The structure of these blood clots provides clues as to how they form and how we can best treat heart attack or stroke patients.

The structure of these blood clots has recently become a hot topic, in conjunction with the emergence of thrombectomy \(^9-12\). Previous approaches using in vivo animal models with intravital fluorescence imaging \(^13\) had a spatial resolution limit that impedes the investigation of the micro-structures of blood clots \(^5\). In contrast, thrombi extracted during a thrombectomy can subsequently be imaged via SEM \(^14-16\) or microscopy of histologic sections \(^10,17\). However, this thrombectomy-based approach has weaknesses. First, extracted thrombi can be deformed or
destroyed during a process of thrombectomy. Second, there is an inevitable time delay between thrombi formation and structural investigation. When a SIPA clot forms in a stenotic artery and blood flow ceases, a coagulation clot may form around the SIPA clot due to the stagnant blood. We hypothesized that occlusion in a stenotic artery is caused by SIPA clot that has a distinctive structure and composition compared to the coagulation clot. The structure will not be homogeneous but show anisotropy from the high shear growth.

In this study, we developed an in vitro flow system that can generate a macroscopic thrombus under arterial hemodynamic conditions that would allow us to retrieve an intact SIPA specimen for the structural investigation. To study the structure of the SIPA clot, we analyzed multiple scanning electron microscopy and histology images. The characteristic structure of the arterial thrombus presented in this study may give insights of the mechanism of occlusion along with possibilities to prevent or treat arterial thrombosis.

**Materials and methods**

**High shear glass tube model**

To generate a SIPA clot, lightly heparinized (3.5 IU/mL) porcine whole blood (400 mL) was perfused through an in vitro flow system. A high flow rate (~1 L/min) was required to generate a high shear rate (> 3,500 s⁻¹) in the glass tube with a stenosis diameter of 2.5 mm. To accommodate this requirement, a closed flow loop was developed with two reservoirs for a constant pressure head (30 mmHg) (Figure 1A). Prior to the blood perfusion, the stenotic region of the tube was coated with 100 μg/ml type 1 fibrillar collagen solution (Chronopar, Chrono-log Inc.) in 0.9% saline by pipetting 60 μl at the stenosis and incubated in a container at room temperature for 24 hours to generate an adhesive surface. The tube was rotated 180 degrees once after an hour, counting from the moment when a collagen solution was added to the tube.
Coagulation clot generation

Porcine blood was treated with 3.2% sodium citrate (10% in volume) during transportation and then recalcified with CaCl$_2$ to a final concentration of 10 mM$^{18,19}$. The recalcified porcine blood was left still for at least 30 minutes at room temperature to form a stable red coagulation clot.

SIPA clot generation in a closed flow loop

Approximately 400 mL of heparinized (3.5 IU/mL) porcine whole blood was used to fill the flow loop and perfused (Figure 1A) to generate a large SIPA clot. A glass tube (Figure 1B) had an inner diameter of 12 mm and 80% stenosis in diameter. The Reynolds number was 334 and the white clot grew from wall to the lumen similar to the capillary tube experiment$^{20}$ where $Re = 162$. The white SIPA clot grew over time at the stenosis (supplementary Movie 1), and the roller pump was manually adjusted to reduce the flow rate and maintain a constant 30 mmHg pressure head (Figure 1C). The maximum shear rate reached over 10,000 s$^{-1}$ with the initial flow rate (Figure 1D), which is in the range where rapid platelet accumulation (RPA) can occur$^{21}$. When the roller pump flow rate reached a minimum level (50 mL/min), the pump was turned off but still maintained the pressure head without significant change. We defined the occlusion time when the pump was turned off. Low flow rate was less than 50 mL/min, and the SIPA clot was allowed to mature. Thus, the circuit was left for an additional 30 minutes to achieve full occlusion. The occlusion was found after draining the blood from the circuit (Figure 1E and F, Supplementary Movie 2), and the generated clot was retrieved from the tube gently. A total of eight SIPA clots were generated in this study and the consistency in gross appearance of all the specimens was remarkable. Two specimens were used for SEM imaging. Three clots were sectioned in longitudinal direction and stained with Carstairs staining and VWF staining. Two clots were sectioned in transverse direction and stained with Carstairs staining and VWF staining.
Computational fluid dynamics (CFD) analysis

Computational fluid dynamics (CFD) was used to quantify the shear rate distribution within the stenotic test section. The steady flow simulation was performed using Ansys 19.1 (Ansys Inc., PA, USA). Whole blood flow was assumed to be a Newtonian fluid of 3.5 cP, and the flow was presumed to be laminar, incompressible, steady, continuum, and isothermal based on the Reynolds number (334) of the experiments. A flow rate of 660 mL/min was applied at the inlet with zero pressure at the outlet. Mesh convergence was achieved at 0.6 million hexagonal cells.

Arterial thrombosis model

SIPA clot growth in the glass tube was predicted using a quantitative, empirical high shear thrombosis model. The model is composed of a lag phase and a rapid platelet accumulation (RPA) phase defined by:

\[ t_{\text{Lag}} = 1.69 \times 10^6 S^{-1.2} \]

Where \( t_{\text{Lag}} \) is lag time and \( S \) is the wall shear rate. Thrombus growth rate during the RPA phase is expressed by time and shear rate (S).

\[ J = \begin{cases} 0, & t \leq t_{\text{Lag}} \\ ae^{bs} + ce^{ds}, & t > t_{\text{Lag}} \end{cases} \]

Constants \( a, b, c, \) and \( d \) are given in Table 1. The lower and upper confidence limit of thrombus growth are denoted by \( J_{\text{MIN}} \) and \( J_{\text{MAX}} \) respectively.

The assumptions of Poiseuille flow were applied at the throat and shear rate was defined by:

\[ S_{\text{Cir}} = \frac{32Q}{\pi d^3} \]
where Q is flow rate and \( d \) denotes channel diameter. An initial flow rate of 0.66 mL/min and diameter of 2 mm at the throat was used and the channel diameter was updated every second as thrombus growth increased in the tube.

**Scanning electron microscope imaging**

The SEM illustrates the morphology and density of the platelets. Blood clots were fixed in a 10% formalin solution for more than 24 hours. A fixed blood clot was submerged into successive ethanol solutions of 25%, 50%, 75%, 80%, and 90% concentrations for 15 min each and rinsed with DI water. After an overnight air dry, the sample was sputter coated with Au and imaged using a SEM (Hitachi SU8230).

**Histology and immunohistochemistry**

The histology revealed the composition and structure of the thrombus. Blood clots were fixed in formalin and embedded in paraffin for further histological and immunohistochemical analyses. The paraffin block holding the clot was cut into 5 µm-thick slices and deparaffinized. Using Carstairs’s staining method \(^{23}\), platelets are stained in light blue, fibrins in red, RBCs in yellow, and white blood cells (WBCs) in black. Separately, VWF immunohistochemistry was conducted using Polink-2 plus DAB (3,3’-Diaminobenzidine) detection system (GBI labs, Mukilteo, WA) and anti-VWF antibody (primary antibody, Biocare medical, Pacheco, CA) \(^{24}\). We added 100 µl or more of primary antibody and incubated in a moist chamber for overnight at room temperature, and later counterstained with Meyer’s haematoxylin.

**Results**

The thrombus developed over an hour to occlusion and blood flow ceased in 60 ± 17 minutes (Figure 1C, \( n = 8 \)). CFD was performed to quantify shear rate at the stenosis (Figure 1D). Using Mehrabadi et al’s high shear thrombosis model \(^{21}\), the flow rate was simulated with the initial
flow rate value from the experimental data (660 mL/min) to estimate the occlusion time to aid in the design of the experiment. The model estimated occlusion at about 71 minutes (Figure 1C) confirming that our microfluidics system creates a similar thrombus to our large-scale system. All occlusive SIPA clots had a similar trumpet-like shape with the mouth opened against the flow (Figure 1F and supplementary Figure 1). After the flow circuit was drained, the SIPA clot was retrieved (Figure 2A) from the large glass tube for SEM imaging and histology.

**Platelets are tightly packed in a SIPA clot at the throat of the stenosis.**

The retrieved gross clot had a similar trumpet-looking shape to the one observed during the blood perfusion (Figure 1E, Figure 1F, Figure 2A, and supplementary Figure 1) and had different colors in characteristic regions. At the upstream and the downstream portions, there were translucent yellow parts that were originally attached to the glass wall (Figure 2B, R5). These parts were thin sheets attached to the upstream region of the stenosis but became folded and tangled during detachment from the wall. We interpret this yellow material to be the gross appearance of adherent VWF. Thrombus recovered from the throat of the stenosis had a light red or pink color (Figure 2B, R2 and R3). This stenotic region was predominantly white clot. Far downstream of the throat was a region of dark red material that looked like coagulated red clot (Figure 2B, R1 and R4). After formalin fixation and dehydration, the clot was cut in half in the flow direction to image the inner part of the clot (Figure 2C). The longitudinal section revealed string-like structures protruding towards the lumen (Figure 2C and D) that were up to 10 times longer than they were wide.

The string or finger-like structure was not preserved in the sectioned SEM images. Geological shapes were seen in the low magnification images (Figure 2E) with layers of aggregates and some fibers. Meanwhile, the moderate magnification images displayed aggregates of numerous spheres that were approximately 2 µm in diameter, which are interpreted as individual, unactivated platelets (Figure 2F); whereas, activated platelets should show spike-like filopodia and spreading disc morphology. The platelet aggregates are easily seen in the high magnification images (Figure 2G). To quantify how many platelets were packed in the clot, recognizable spheres with a diameter greater than 1.5 µm were manually counted in...
calibrated sections of the high magnification SEM images. Using the observed 2 µm platelet thickness on the SEM images with an average value of 0.1 platelets/µm² (Figure 2H), we calculate a platelet density per volume as 0.05 platelets/µm³ and the total SIPA clot is estimated to contain 1.23 billion platelets.

The SIPA clot occludes from strings of platelets and VWF coalescing with a porous structure.

SIPA clots were stained using the Carstairs or VWF immunostaining method. Three clots were sectioned in a longitudinal direction, and the other two clots were sliced in a transverse direction. Consistent with the SEM results, Carstairs staining showed that the SIPA clot was dominantly occupied by platelets (blue, Figure 3A and Figure 4A to I) and immunostaining result displayed that VWF was present throughout the SIPA clot (Figure 5), proving that the SIPA clot is VWF and platelet-rich opposed to coagulation clots (control, supplementary Figure 2 and 3). Meanwhile, although the coagulation cascade is unlikely due to pathological high shear conditions, Carstairs staining did reveal a few RBCs (yellow to orange) and some fibrin (red) in the clot (Figure 3). Presumably, RBCs become trapped and fibrin forms in stagnant pockets within the overall platelet thrombus or after occlusion formation. Thus, the void region and the space occupied by RBCs and fibrin are assumed to be pores or valleys that were formed during SIPA clot formation.

The histological appearance reflected the gross appearance seen macroscopically, as is seen in Figure 2C. The transparent, thin sheets in the far upstream region was dominantly VWF with few platelets, but also contained some fibrin (Figure 3B). The small, dispersed platelet aggregates were surrounded by fibrin (Figure 3B). As the stenosis converged, the platelets and trapped RBCs formed a stripe-like pattern (Figure 3A and C). The platelet fingers alternated with stripes of RBCs that were trapped in the space between the platelet aggregates (Figure 3C). Moving into the throat of the stenosis where the shear rate and SIPA were expected accelerate, this zone had axisymmetric structures with few trapped RBCs. Fibrin was visible in pores near the center of the lumen (Figure 3A and D). A thin zone of light blue can be seen just next to the wall representing fewer platelets attached to the wall. Then a color gradation of dark blue to
medium blue extended to the center of lumen representing dense platelets at the periphery and less dense platelets at the center (Figure 3A). Downstream of the stenosis in the expansion region, the core protruded in the center, but the thrombus separated from the wall, consistent with a flow separation zone seen in the CFD Figure 1D. The separation zone was filled with loose RBCs, but not a platelet mass (Figure 3E).

Another SIPA clot was sectioned in a transverse direction and stained using the Carstairs and VWF immunostaining method. The upstream section shows a large number of trapped RBCs and a small number of dispersed platelet aggregates with fibrin (Figure 4A) and VWF (Figure 5A). The next section shows a large hole at the center and trapped RBCs and some fibrin in the pores (Figure 4B). The stenotic sections reveal thrombus that completely occludes the lumen by a mass of blue platelets interspersed with pores filled with RBCs (Figure 4C to G). Further downstream past the stenosis, there is a bull’s eye or target appearance with a central dense core surrounded by a layer of loose RBCs and then a thin layer of platelets at the wall (Figure 4H and I). As it gets close to the throat, the platelet proportion increases but fibrin proportion decreases (Figure 4J).

Summarizing the findings from histology and SEM, the occluding throat section is composed of 80% dense platelets and VWF with 5% fibrin and many channels ranging from 10 to 100 µm. The upstream region showed long VWF strands adherent to the wall without large platelet aggregates where shear rates are less than 1,400 s⁻¹ (white arrows in Figure 2D). Stripes of platelet aggregates and individual RBCs were found in upstream and downstream regions, where more unactivated platelets are seen on SEM than the stenosis region (Figure 2G from the downstream throat R4 in Figure 2B and Supplementary Figure 4 from the throat R3 in Figure 2B). Platelets within the stenosis throat were mostly misshapen consistent with prior activation by the very high shear rate up to 9,000 s⁻¹ (Figure 1D). The downstream end of the thrombus on SEM showed a protruding central core of round platelets that appear not to have activated. Surrounding the core was an annulus without thrombus where there would be flow separation with lower shear rate. At the wall is a thin ring of VWF and small aggregates hugging the collagen surface and extending throughout the recirculation region.
The SIPA clot is rich in VWF, especially near the wall

Staining for VWF (brown) is found throughout the SIPA clot which is not present for PRP clot formed under stagnant conditions (supplementary Figure 3). In the longitudinal and transverse directions, the immunostaining results showed that VWFs were concentrated at the edge where the clot adjoined the wall (Figure 5). In contrast, in the Carstairs staining result, the blue coloring was lighter near the wall, indicating that fewer platelets aggregated at the wall during the lag phase (Figure 4). This complementary density of VWF and platelet was quantified in Figure 6, which plots normalized intensity level of VWF and platelet from wall to the lumen. Therefore, when the SIPA clot started to develop, a large amount of VWFs must have accumulated at the wall first in order to capture the marginated platelets. The thickness of this VWF-rich layer was about 20 µm, which had fewer platelets.

Discussion

SIPA clots were generated in a closed in vitro flow loop to investigate the SIPA clot structure. The size of the generated SIPA clot was anatomically relevant to diseased arteries (2 mm in diameter and 5 mm in length) and had a trumpet-like shape with the horn facing upstream. This SIPA clot is 2 to 20-fold larger in diameter compared to previously generated SIPA clots,\textsuperscript{26,27} which allowed us to collect the clot with the structure intact. We were able to section the specimens in the longitudinal or transverse direction. SEM images showed that the platelets were tightly packed in the clot with a density of 0.1 platelets/µm\textsuperscript{2}, indicating that the 1.23 billion platelets were aggregated in the SIPA clot, which is significantly different from previous images of RBC-rich blood clots.\textsuperscript{14-16} Spherical platelets were found in the SEM images, and its morphology was similar to the platelets found in the ultra-densely packed region of mice puncture injury model by Tomaiuolo, et al.\textsuperscript{28} The histological images showed consistent results in which the clot was VWF-platelet-rich, and RBCs were trapped in the pores, which is strikingly similar to SIPA clots formed in our mice arterial thrombosis model.\textsuperscript{29} The high platelet content of our SIPA clot found from both SEM images and histological images are also consistent with composition of arterial clots found from clinical studies.\textsuperscript{5,8} Our SIPA thrombi look
very similar to the platelet rich clinical clots in Staessens et al\textsuperscript{8} showing platelet content between 90\% to 50\% as well as VWF staining. Our SIPA clots appear consistent with the platelet-rich regions seen in the clinical blood clot images shown in papers by Tutwiler et al\textsuperscript{30}, Staessens et al\textsuperscript{10}, and Chernysh et al\textsuperscript{16}. Note that our fresh thrombi were not given time to contract into a mature clot. The clinical samples are retrieved without orientation and may represent a mixture of central white clot mixed with coagulation clots upstream and downstream formed by stagnant blood after occlusion. Our thrombi show additional spatial detail than harvested pieces of clinical thrombus. Nonetheless, our clots do show consistency with the retrieved clinical clots that are platelet-rich.

The SIPA clot structure revealed here may be explained by a 7-step process for a SIPA clot formation\textsuperscript{31}. The process starts with high wall shear rates (step 1) and VWF adsorption onto the collagen surface (step 2, Figure 7-1). In this study, the SIPA clot had a high VWF density on the wall, but sparse platelets compared to the lumen side (Figure 7-2). The plasma VWF must be important for the lag phase (step 1-2), as platelets need to be captured at the wall (step 3-4). Casa et al.\textsuperscript{32} found that plasma VWF is more important than platelets in forming occlusive SIPA clots. When a sufficient amount of insoluble VWFs are accumulated at the wall, a number of platelets are captured (step 3-4) and activated. These mural platelets can make new highly reactive surfaces by releasing \(\alpha\)-granules of VWF (step 5). VWF release from \(\alpha\)-granules are necessary to initiate the RPA phase (step 5-7) and the subsequent vessel occlusion\textsuperscript{29}. The histological images show that there was a marked transition from light blue to dark blue all the way to the lumen. This light blue layer was about 20 \(\mu\)m thick, which is comparable to the thrombus thickness hypothesized by Bark, Para, and Ku\textsuperscript{33} for the lag phase as a transition from the lag phase to the RPA phase. Between the 20 \(\mu\)m wall layer and center of the lumen, string-like structures protrude from wall to the lumen were found in longitudinally cut SIPA clot (Figure 2C) and Carstairs staining slice in a longitudinal direction (Figure 3A). This string-like structure has been noted by previous investigators for VWF + platelets and was prominent at the upstream section (Figure 2B, R1), with RBCs trapped between the fingers. Past studies also observed these SIPA clot structures, which was referred to as “fingers” in expanded polytetrafluoroethylene vascular graft tubing\textsuperscript{26} and glass capillary tube\textsuperscript{27}, but this is the first time to
report string-like structure in an intact, oriented occlusive SIPA clot. Inhibiting \( \alpha \)-granule release may interrupt the long VWF + platelet fingers, thus preventing vessel occlusion \(^{29}\).

These fingers grew from the wall towards the center of the lumen to occlude the large (~2.5 mm in diameter) channel (Figure 7-3). The fingers appear to have been growing faster near the apex due to a higher shear rate that promotes thrombus growth rate \(^{21}\) (Figure 7-4). The faster growth at the apex region can constrict the lumen, creating a recirculation area similar to Kim, et al. \(^{20}\) observed in their capillary tube model that arrests SIPA distally (Figure 2B, R4). Later, the fingers in apex region coalesce, occlude the channel, and stop blood flow, which inhibits the proximal region to grow and occlude the channel (Figure 7-6). The platelet aggregates that were captured near apex may extend downstream (Figure 2B, R4). Fibrin may form at the pores after the SIPA clot occluded the lumen, thus lowering the shear rate and enabling the coagulation cascade to occur (Figure 7-7). These whole processes are illustrated in Figure 7. In contrast, the proximal section (Figure 2B, R1) continues to grow but does not bridge the larger lumen size (Figure 7-5). The resulting SIPA clot resembles the SIPA clot from smaller glass capillary tube \(^{7,34}\); however, the specific finger-like platelet aggregates extending into the lumen and the porous structure are new findings in this paper.

The pores observed in the transverse clot section imply that the SIPA clot create a high permeability as was predicted by Du et al \(^{35}\) and would allow convective drug transport through the inner clot \(^{36}\). Although a drug could be transported to the center of the clot through the pores, tPA may not work on a SIPA clot where the histological images only trace amounts of fibrin. Indeed, Kim, et al. \(^{20}\) found DiNAC (diacetyl-L-cystine) lyses the SIPA clot better than tPA. Thus, perfusing a thrombolytic agent that reacts with VWF or platelets may be needed to dissolve a SIPA clot \(^{20}\).

The present study had several limitations. First, only one animal’s (porcine) blood was used to generate the clot. However, Para and Ku \(^{27}\) found there was no significant difference between human and porcine blood when generating a SIPA clot. Second, we used a glass tube, which had rigid impermeable walls, and steady flow was applied for the SIPA clot generation. With the alternative triad for SIPA clot formation, we generated a SIPA clot under a controlled in vitro flow system and the clot was retrievable without any structural damage. This system can be used to generate SIPA clots for testing thrombolytic agents or thrombectomy devices. The SIPA clot structure found in this study may be used to recreate clots collected through thrombectomy.
Data Sharing Statement
For data sharing, please contact the corresponding author at david.ku@me.gatech.edu.

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Authorship Contributions
D.A.K. and D.N.K designed research. D.A.K. performed the research. All authors wrote the paper.

Conflict-of-interest disclosure
The authors declare no competing financial interests.
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Table 1. Constants for RPA phase thrombus growth rate equation for the empirical model.\textsuperscript{21}

|       | \( A \)  | \( b \times 10^{-4} \) | \( c \)  | \( d \times 10^{-6} \) |
|-------|----------|--------------------------|---------|--------------------------|
| \( J_{\text{MIN}} \) | -28.3    | -1.00                    | 27.4    | -10.0                    |
| \( J_{\text{AVG}} \) | -31.3    | -1.45                    | 30.7    | -6.81                    |
| \( J_{\text{MAX}} \) | -38.2    | -1.81                    | 36.6    | -5.92                    |

Figure 1. \textit{In vitro} flow loop for a SIPA clot generation. (A) A constant-pressure closed loop circuit with a roller pump was developed to generate a clot in (B) a collagen-coated glass stenosis that has a throat diameter of approximately 2 mm. A total volume 400 mL of blood was circulate through the flow loop. (C) To maintain a constant 30 mmHg pressure head, the roller pump flow rate was reduced as the clot grew over time (red, average of \( n = 8 \) and standard deviation shown in bars) as compared to the simulated flow rate in an empirical model of SIPA thrombus growth rate (blue, upper and lower limit displayed with bars). (D) CFD showed the initial maximum shear rate in the throat was greater than 10,000 s\(^{-1}\). The area of high shear is consistent with the thrombus location in the stenosis. (E) The SIPA clot occluded the stenosis with a trumpet-like shape. The boundary of the SIPA clot is highlighted by the yellow dotted line. (F) The glass tube with the SIPA clot after blood was drained, showing extension of the thrombus at the wall both upstream and downstream of the throat.

Figure 2. Harvested SIPA clot and SEM images. (A) A SIPA clot retrieved from the large glass tube was formalin fixed and dehydrated using ethanol. (B) Schematic of a SIPA clot with ROIs (R1 to R5). (C) A SIPA clot cut in half to expose cross section. (D) Platelet-rich white region is highlighted in green showing fingers of platelet aggregates (white arrows). (E-G) SEM image of SIPA clot ROI R4. (E) Low magnification SEM image of the SIPA clot showing the dense core of the thrombus. (F) Moderate magnification SEM image of the SIPA clot illustrating a textural surface of platelet aggregates. (G) High magnification SEM image of the SIPA clot showing amorphous platelets that are likely activated with degranulation. (H) Platelet density from SEM images of regions R1 to R5.

Figure 3. Carstairs staining of a SIPA clot in the longitudinal direction. (A) The trumpet-like morphology of the gross clot was preserved. (B) and (F) Each end of the clot showed small platelet aggregates with fibrin. (C) Upstream of the throat, the thrombus had platelet
aggregates protruding towards lumen with string or finger-like shapes alternating with trapped RBCs. Some strings were 10x longer than they were wide. (D) Apex of stenosis was fully occluded with platelets. Some fibrin was found in the center of the lumen and pores. (E) Downstream, the clot was present at the center and near the wall; whereas, the annulus between the two had sparse RBCs and fibrin without platelet aggregates. R1 to R5 indicates the ROI indicated in Figure 2B.

Figure 4. Carstairs staining of the SIPA clot in the transverse direction. Starting from the upstream, each section is spaced approximately 0.5 mm apart. R1 to R4 indicates the ROI indicated in Figure 2B.

Figure 5. VWF immunostaining of the SIPA clot in the transverse direction. Starting from the upstream, each section is spaced approximately 0.5 mm apart. R1 to R5 indicates the ROI indicated in Figure 2B.

Figure 6. (A) VWF staining and (B) Carstairs staining of a SIPA clot in the transverse direction (10x magnification). (C) Normalized intensity level along the red lines in (A) and (B). Near the wall, VWF was concentrated and dark, yielding low intensity levels; whereas, Carstairs platelet was light at the wall corresponding to a high intensity level. Black arrows indicate the starting point close to the wall and blue arrows indicate a pore in the clot.

Figure 7. Process of SIPA clot formation in a large stenotic vessel. 1) Schematic shows bottom half part of stenosed vessel. Dotted line indicates the axisymmetric centerline. VWF (brown) adsorption onto the collagen surface under high shear rate condition. 2) Sparse platelet adhesion (light blue) to the surface. 3) Displays red dotted box in 2). Rapid platelet aggregation (RPA) begins and forms mountains and valleys of platelet aggregates. 4) Shear-induced platelet aggregation (SIPA) accelerates the thrombus growth with higher shear rate near the apex. 5) Growing thrombus at the apex alters the flow environment and creates larger recirculation region downstream. 6) SIPA clot occludes the vessel. 7) Final SIPA clot (light grey) comparison between schematic (left) and experimental picture (right).
Figure 2

A. Image showing a brownish object with a "FLOW" indication.

B. Diagram with labeled regions R1 to R5.

C. Image of a different brownish object.

D. Image with green and brownish areas.

E. SEM micrograph showing a texture.

F. SEM micrograph at a higher magnification.

G. High-resolution SEM image.

H. Bar graph showing platelet density with values for R1 to R5.
Figure 3
Figure 4

A: R1a  B: R1b  C: R2a
D: R2b  E: R3a  F: R3b  G: R3c
H: R4a  I: R4b

J

| Location | Platelet | Fibrin | RBC | Void |
|----------|----------|--------|-----|------|
| R1a      |          |        |     |      |
| R1b      |          |        |     |      |
| R2a      |          |        |     |      |
| R2b      |          |        |     |      |
| R3a      |          |        |     |      |
| R3b      |          |        |     |      |
| R3c      |          |        |     |      |
| R4a      |          |        |     |      |
| R4b      |          |        |     |      |
Figure 5

A: R1a  B: R1b  C: R2a  D: R2b
E: R3a  F: R3b  G: R3c  H: R4a  I: R4b
Figure 7

1. VWF adsorption

2. Platelet adhesion

Platelets

3. RPA – Mountains and valleys

Platelet aggregates

4. Shear induced platelet aggregation

Higher shear → Faster growth

5. Clot alters flow environment

6. Occlusion

Growth↑

Flow

Recirculation

7. Coagulation in stagnation area (Orange)