Microclot array elastometry for integrated measurement of thrombus formation and clot biomechanics under fluid shear

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Blood clotting at the vascular injury site is a complex process that involves platelet adhesion and clot stiffening/contraction in the milieu of fluid flow. An integrated understanding of the hemodynamics and tissue mechanics regulating this process is currently lacking due to the absence of an experimental system that can simultaneously model clot formation and measure clot mechanics under shear flow. Here we develop a microfluidic-integrated microclot-array-elastometry system (clotMAT) that recapitulates dynamic changes in clot mechanics under physiological shear. Treatments with procoagulants and platelet antagonists and studies with diseased patient plasma demonstrate the ability of the system to assay clot biomechanics associated with common antiplatelet treatments and bleeding disorders. The changes of clot mechanics under biochemical treatments and shear flow demonstrate independent yet equally strong effects of these two stimulants on clot stiffening. This microtissue force sensing system may have future research and diagnostic potential for various bleeding disorders.
Blood clotting at the vascular injury site is a complex biomechanical process mediated by several key factors including platelet deposition and contraction, clot stiffening and hemodynamic forces. In hemostasis, the interplay of these mechanical factors leads to strong clots that stem bleeding, but in clotting disorders such as von Willebrand disease (VWD), disrupted biomechanical interactions lead to weak clots and bleeding. Although different aspects of the clotting problem such as shear flow-mediated platelet adhesion and single platelet mechanics have been studied in the past, an integrated understanding of the interplay between clot mechanics and hemodynamic shear is still missing. This is mainly due to the lack of combined capability to model and measure clotting mechanics in the presence of shear flow in existing research models.

Recent technological advancements have resulted in the evolution of independent approaches that focus on different parts of the clotting problem. For example, microfluidics systems create well-controlled blood flow in matrix protein-coated microchannels, and thus they have become a widely used vascular injury model for the study of platelet adhesion, aggregation, and thrombus formation. These systems are powerful tools to study the effects of various flow conditions, surface chemistries and coagulation factors on platelet adhesion and aggregation, however, they are not equipped with the ability to measure clot mechanics. Furthermore, matrix proteins are coated on glass or relatively rigid surfaces, which do not mimic the physiological stiffness of exposed matrix at vascular injury sites. In the study of platelet mechanics, atomic force microscopy and flexible microspot arrays have been used to measure single platelet-generated contractile forces under static conditions, and matrix protein microdot arrays have been used to study the effects of substrate stiffness and mechanosensitive signaling pathways on platelet-generated contractile force. Single platelet-mediated fibrin fiber remodeling has also been studied using confocal microscopy. While these studies provide important information on platelet mechanics, they do not include the effect of hemodynamic forces and offer limited insight into clot mechanics at the tissue level. In the clinic, cup and cone type of rheometers, also known as thromboelastography (TEG) or rotation thromboelastometry (ROTEM), have been developed to measure clot stiffness at different stages of the coagulation process. Although these systems provide useful information that can assist the diagnosis of coagulation disorders, their consideration of hemodynamic flow and platelet–matrix interactions is inadequate. As a result, their diagnostic power is limited and they are seldom applied for disorders of primary hemostasis, like VWD. Together, the lack of an integrated system that can follow clot formation and mechanical properties in the presence of shear flow has hindered an integrated understanding of the biomechanical events occurring during blood clotting.

With recent advent of microfabrication technology, we and others have created microtissue array-based mechanical sensing platforms that allow simultaneous control of tissue formation and measurement of tissue mechanics. In this system, cell contraction-mediated self-assembly of matrix proteins such as collagen and fibrin leads to the formation of submillimeter microtissues that anchor on top of multiple flexible poly(dimethylsiloxane) (PDMS) micropillars. These micropillars serve as mechanical sensors that can report in-situ tissue mechanical properties. The composition and stiffness of these microtissues, contributed both by preseeded and cell-secreted matrix proteins, mimic those of native vascular tissue. By virtue of their small dimensions, the microtissues offer orders of magnitude scale-up advantages over conventional bulk hydrogel models and open up the possibility to integrate with microfluidics. This system has been used to study the mechanics of cardiac and skeletal muscles, fibrotic tissues and wound healing; however, it has not been integrated with the shear flow.

In the current study, we develop a microfluidic-integrated microclot array elastometry (clotMAT) system by integrating a collagen microtissue array-based mechanical sensing platform with platelet flow. This system recapitulates the dynamic process of platelet adhesion and clot formation under various flow conditions and reports changes in clot mechanical properties in real time. Through platelet agonist (ADP and thrombin) treatments, we dissected the contributions of hemodynamic and tissue/cell forces to clot stiffening. Treatments with platelet antagonist (glycoprotein receptor antibodies, blebbistatin and acetylsalicylic acid (ASA)) further enabled the examination of the effects of platelet adhesion and cytoskeletal tension on clot mechanics. The utility of the clotMAT system in bleeding disorders was demonstrated through the study of clotting mechanics of VWD. Integrated analysis of results show strong correlation between clot stiffness, clot contractile force and the level of clot retraction (volume shrinkage) under both biochemically treated conditions and shear flow conditions, suggesting these two stimulants independently yet equally strongly affect clot remodeling and stiffening. This finding unveils the tissue remodeling/stiffening mechanism in a previously unexplored shear flow regime, and thus significantly expands the boundary of previously established tissue remodeling theory. Together, this work describes a physiologically relevant microtissue force sensing system for the study of normal and abnormal clot biomechanics under shear flow, which may have future research and diagnostic potential for various bleeding disorders.

**Results**

**Microclot array formation under platelet flow.** The clotMAT device consists of three functional layers including a top PDMS layer containing multiple microfluidic channels for platelet flow, a middle PDMS layer containing arrays of collagen microtissues that capture flowing platelets and report clot contractile forces, and a bottom stretchable silicone membrane for clot stiffness measurement. An array of submillimeter-size microwells (13 columns by 6 rows) were cast in the middle PDMS layer, and each microwell contains a pair of flexible PDMS micropillars that serve as force sensors to report clot-generated contractile forces. A single micropillar consists of a slim leg section and an enlarged head section that provides mechanical constraint to the suspended microtissue (Supplementary Fig. 2). The collagen microtissue array was fabricated by utilizing endothelial cell-mediated collagen gel compaction (Supplementary Movie 1). Additional steps were performed to remove originally seeded endothelial cells after dog-bone-shaped microtissue formation (Supplementary Fig. 1), in order to recapitulate the matrix composition anticipated during vascular injury. The resulting individual bare micro-collagen matrix hangs between the heads of a pair of flexible micropillars (Supplementary Figs. 2, 3), mimicking exposed subendothelial collagen at vessel injury sites and avoiding the effect of rigid substrates used in existing microfluidic devices. The bare collagen microtissues measure 132.7 ± 17.2 µm (mean ± SD, 113 replicates) wide and 80.7 ± 5.9 µm (16 replicates) thick. The microfluidic channels in the top PDMS layer were aligned with individual rows of collagen microtissues in the middle layer (Supplementary Fig. 4).
When the microchannels were perfused with citrated platelet-rich plasma (PRP), flowing platelets were arrested on a row of individual micro-collagen matrices contained by the microchannel (Fig. 1c–e). The adhered platelets gradually covered the collagen matrix, as demonstrated by the gradual increase in fluorescence intensity of the microtissue (Supplementary Movie 2). Over a period of 30 min, platelets continued to aggregate and fibrin continued to polymerize over the collagen matrix surface, leading to the formation of a submillimeter-sized microclot hanging between two micropillars (Fig. 1f, g). SEM and confocal imaging of microclots formed using healthy PRP show that aggregated platelets and fibrin meshwork together formed a thick clot shell covering the bare collagen core. The width of the whole microclot is $83.3 \pm 9.2 \mu m$ (14 replicates). The thickness of the clot shell is $28.2 \pm 7.6 \mu m$ (14 replicates) and that of the whole clot is $64.1 \pm 5.8 \mu m$ (14 replicates, Supplementary Figs. 5–7). Compared to the bare collagen matrix before flow, the size of the collagen core decreased after platelet flow. Such compaction of the matrix is contributed by the collective contraction of activated platelets, as detected by the deflection of the micropillars (Fig. 1f, g).

**Modeling clot contraction and stiffening by microclots.** Upon clot formation, activated platelets generate contractile forces that lead to clot retraction. To detect the minute change in microclot-generated contractile forces, micropillars were engineered to sense force in nanoNewton range ($k = 120 \text{nN} \mu \text{m}^{-1}$, Supplementary Fig. 2), and micropillar head displacements ($\delta_1$ and $\delta_2$) were used to calculate contractile force according to cantilever bending theory (Fig. 2a). Here, we observed that micropillars were sensitive enough to detect the increase in clot contractile force upon platelet flow, as demonstrated by the obvious inward deflection of the microclots (Figs. 1f, 2b, Supplementary Fig. 8). Such increase in contractile force is...
accompounded by significant increase in tissue-level fluorescence intensity (Fig. 2c, d), confirming that contractile force is mainly generated by aggregated platelets. In contrast, shear flow without platelet has negligible effect on measured contractile force (Supplementary Fig. 9).

During clot remodeling, progressive clot retraction is often accompanied by clot stiffening. In the clotMAT system, microclot stiffness was measured by tensile testing, which was enabled by stretching the bottom silicone membrane (Fig. 2f, Supplementary Fig. 10a–c). Externally applied tensile force was reported by micropillar deflection as \( F = k(\delta_1 + \delta_2)/2 \), and the microtissue tensile strain was determined based on image tracking of the displacement of fiducial markers in the microtissue before and after stretch (Supplementary Fig. 11). The stress and strain relationship derived from tensile testing results was then used to calculate microclot stiffness (Supplementary Fig. 10d). Under the same amount of substrate stretch, the forced elongation of the microclot (\( \delta_{1\text{micro-clot}} + \delta_{2\text{micro-clot}} \)) was less than that of bare collagen microtissue (\( \delta_{1\text{collagen}} + \delta_{2\text{collagen}} \)) (Fig. 2f), indicating higher resistance to the stretch and thus higher stiffness of the microclots as compared to that of the bare collagen microtissues. The stiffness of the bare collagen matrix (5.7 ± 0.8 kPa, 36 replicates) falls within the range that has been shown to be optimal for platelet adhesion and activation, and thus it allows the modeling of platelet adhesion on physiologically soft subenthothelial matrix (Fig. 2g). The stiffness of microclots (18.0 ± 2.8 kPa, 34 replicates), formed under 30 min of citrated PRP flow at 500 s⁻¹ shear rate, is much higher than
that of the bare collagen matrix and agrees well with the stiffness of laboratory-created thromboembolic analogs. Such difference in measured stiffness suggests that the clotMAT device is sensitive enough to detect the stiffness increase occurred during microclot formation and remodeling.

In addition to the standard sample preparation condition (citrated PRP), we also tested the utility of the clotMAT system under several other common preparation conditions, including the use of whole blood, recalified PRP samples and different anticoagulants. Since red blood cells (RBCs) interfere with optical detection, we washed away unbounded RBCs at the end of 30 min of whole blood flow at 500 s⁻¹ shear rate and measured the microclot properties (Supplementary Fig. 13). Here, we observed that the size of the microclot formed with whole blood is larger and the contraction force is higher as compared to the microclots formed with PRP. Recalcification of citrated PRP significantly increased platelet adhesion, leading to the formation of a large, loose clot that hung above the microtissue and blocked the channel after 10 min of flow. Due to the relatively short flow period, we did not detect significant difference in the contraction force between microclots formed under citrate PRP vs. recalci- PRP (Supplementary Fig. 14). We also compared the properties of microclots formed under different anticoagulants. PRP samples were prepared using either sodium citrate or d-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (70 μM PPACK, a thrombin inhibitor). Here, we observed more platelet binding and higher contractile force generation in the early stage (first 10 min) of flow experiment in the PPACK group compared to the citrate group (Supplementary Fig. 15). Even though platelets are robustly activated by collagen regardless of the anticoagulant (Supplementary Fig. 16), PPACK restricts thrombin-mediated fibrin formation (Supplementary Fig. 17). The lack of fibrin commonly resulted in platelet detachment/thromboembolism in the PPACK run and this is observed as a decrease in platelet fluorescence intensity and contractile force in the mid to late stage (10–30 min) of the flow experiment in the clotMAT system (Supple- mentary Fig. 15)³¹. Treatment of the PPACK group with thrombin receptor activator peptide 6 (TRAP 6) rescued the decrease in fluorescence intensity during mid to late stage of the flow experiment in the clotMAT system but had no effect on contractile force (Supplementary Fig. 15). Overall, while fibrin formed by locally released thrombin from platelets may contribute to clot formation, stabilization and part of the contraction, contractile forces generated by the activated platelets and transmitted via direct adhesive interactions between platelets and the collagen matrix may be the primarily driver of clot contraction. Additionally, these results demonstrate the ability of the clotMAT system to model and measure clot formation under various coagulation conditions.

**Microclot mechanics under various shear flow conditions.** As a demonstration of the integrated modeling and measurement capacity of the clotMAT system, we studied clot mechanics under various shear flow-mediated clotting conditions. Thus, fluid shear rate was varied from 3 to 1500 s⁻¹, and microtissue contractile force, fluorescence intensity, stiffness and tissue volume were measured over 30 min of citrated PRP flow (Fig. 3a). For all the shear rates tested, we observed that microtissue contractile force and fluorescence intensity continuously increased over the 30-min period (Fig. 3b–d). However, microtissue width and topview area continuously decreased during the same period (Supple- mentary Fig. 18a, b). The changing rate of these parameters (i.e. the slope of time-dependent curves) increased with increased flow rate (Fig. 3b–d, Supplementary Fig. 18a, b), indicating a shear-rate-dependent alteration in microclot properties. Indeed, comparison of results between different flow rates revealed that tissue fluorescence intensity, microtissue contractile force and microtissue stiffness increased with increased shear rate (Fig. 3e–g), but microtissue thickness and volume decreased with increased flow rate (Fig. 3h, Supplementary Fig. 19). Together, the data indicate that increased shear rate promotes platelet adhesion, microtissue contractile force generation, volume retraction and stiffening. Such shear-rate-dependent changes in microtissue mechanics may be due to the nature of shear-rate-dependent platelet adhesion.

**Platelet agonist and antagonist treatments on microclots.** We biochemically characterized the clotMAT system using a range of agonists/procoagulants and antagonists (Fig. 4a). First, in order to dissect the contributions of platelet number and the contractility of individual platelets to clot stiffness and contractility, we perfused 10 μM ADP or 5 U mL⁻¹ thrombin over microclots that were previously formed under 30 min flow of citrated PRP at 500 s⁻¹. Since platelets were absent in the agonists-containing perfusion buffer, the number of recruited platelets was compara- ble between this experiment and the standard flow condition. Consistent with this notion, neither ADP nor thrombin caused significant change in the microclot fluorescence intensity (Fig. 4b). However, both stimuli significantly increased microtissue contractile force and stiffness, and decreased microtissue volume (Fig. 4c–e, Supplementary Fig. 20), with the effects of ADP being greater compared to thrombin under the conditions tested. The stiffness of ADP-treated microclots (28.4 ± 6.1 kPa, 22 replicates) was 56% higher than that of untreated microclots and agrees well with the stiffness of fresh thrombi (26 ± 2.6 kPa) collected from carotid and cerebral arteries in stroke patients. Since the above changes in the clot mechanical properties were not due to alterations in platelet number, these data confirm that individual platelet-generated contractile force, rather than simply platelet number, contribute to the measured changes in clot stiffness and volume. The more profound effect of ADP (MW 427 Da) compared to thrombin (MW 37 kDa) in this setting may be due to its smaller size which enables greater penetration into the platelet- dense clot, unlike thrombin which would be diffusion limited. Indeed, when thrombin or ADP was added to citrated PRP just prior to flow, thrombin caused the formation of larger, fibrin-rich clots that blocked the flow channel within minutes (Supplementary Fig. 21).

To determine the molecular players regulating clotting in this model, we applied antagonists against the platelet glycoprotein receptor GpIbα epitope (mAb HIP1) that recognizes von Willebrand factor (VWF), and also a GpIIb-IIIa receptor antagonist (Abciximab) at both venous (500 s⁻¹) and arterial (1500 s⁻¹) shear rates. Here, antagonist treatment significantly inhibited platelet binding to the collagen matrix and associated fluorescence intensity (Fig. 4f, g). Reduction in microtissue contractile force and stiffness (Fig. 4h, i) and increase in microtissue volume (Fig. 4j, Supplementary Fig. 20) were also observed under these treatments as compared to untreated PRP, indicating lack of functional platelet activity. Among the antagonists, inhibition of GpIIb-IIIa function by Abciximab blocked platelet recruitment, clot contraction and stiffening at all shear rates while the anti-GpIbα mAb (HIP1) was more effective only at the higher shear rate, 1500 s⁻¹ (Fig. 4f–j). This is consistent with the known property of GpIbα–VWF interaction in the arterial shear regime. Together, these observations are consistent with the notion that initial platelet recruitment is facilitated by GpIbα binding to VWF that is initially recruited onto the collagen matrix. This is likely followed by the engagement of GpIIb-IIIa and other proteins that engage platelets
onto the extracellular matrix\textsuperscript{36,37}. The colocalization of fibrin and platelet signals in confocal images suggests that platelet adhesion and fibrin formation occur over overlapping time scales (Supplementary Figs. 5 and 6).

In addition to platelet antagonists, we also used a myosin IIA inhibitor blebbistatin to simulate platelet myosin IIA impairment-induced clotting disorder, such as those seen in May-Hegglin anomaly, Fechtner, Epstein, and Sebastian
syndromes. Blebbistatin treatment in citrated PRP flow for 30 min at 500 s$^{-1}$ shear rate resulted in faint fluorescence (Fig. 4f, g), a significant reduction in microtissue contractile force and stiffness (Fig. 4h, j), and an increase in microtissue volume (Fig. 4j, Supplementary Fig. 20), as compared to untreated PRP sample. Such impairment on the microcot mechanical properties by blebbistatin is consistent with its effects on disrupting thrombus consolidation and stability reported in the previous study. To examine the diagnostic potential of the clotMAT system, we tested the response of the clotMAT system to acetylsalicylic acid (ASA, aspirin) treatment. ASA treatment of citrated PRP resulted in significant reductions in microtissue fluorescence (Fig. 4f, g), microtissue contractile force, stiffness (Fig. 4h, i), and an increase in microtissue volume (Fig. 4j, Supplementary Fig. 20), as compared to untreated PRP sample, which is consistent with previous study. Overall, these data demonstrate that the clotMAT mimics molecular aspects previously reported in classical flow chamber/microfluidics systems in collagen-based substrate assays; only now we can also simultaneously measure platelet contractility and clot stiffness.

**VWD patient sample results in mechanically weak microclots.** We determined if the clotMAT system can be used for the diagnosis of bleeding disorders. To this end, we used VWD (von Willebrand Disease) type 2A patient plasma due to the prevalence of this disease and the role of VWF on platelet adhesion in this system (Fig. 5a). In these patients, enhanced ADAMTS13-mediated proteolysis results in deficiency in high molecular mass VWF multimers, lower plasma VWF concentrations and reduced shear-induced platelet activation (Supplementary Fig. 22). Flow experiments using reconstituted VWD PRP (rVWD PRP) for 30 min at 500 s$^{-1}$ resulted in faint fluorescence in microtissues, indicating significant reduction in platelet binding to collagen (Fig. 5b, c). Microtissue contractile force and stiffness were also lower and microtissue volume was higher in reconstituted VWD sample as compared to control samples including both healthy PRP and reconstituted healthy PRP (rHealthy PRP) (Fig. 5d–f). Together, these results demonstrate that the clotMAT device is sensitive enough to detect the change in clot mechanics associated with bleeding disorders.

**Effects of biochemicals and shear flow on clot stiffening.** The ability of the clotMAT system to report major mechanical parameters involved in clotting process enables us to perform integrated, quantitative analysis of these parameters. Using data collected from different clotting experiments performed under various flow and coagulation conditions, we performed regression analyses of the microcot contractile force against its stiffness and volume. We then compare the regression slopes between flow-mediated condition and biochemically induced coagulation conditions. Here microcot volume is used as a quantitative measurement of the clot retraction level. For biochemically induced coagulation conditions (procoagulation factors, antagonists, VWD patient sample), we observed strong correlations between microcot stiffness and its contractile force ($R^2 = 0.97$, Fig. 6a, Supplementary Fig. 23) and between microcot volume and its contractile force ($R^2 = 0.93$, Fig. 6b). For all shear rates tested (3–1500 s$^{-1}$ shear rate), strong correlations between these mechanical parameters were also observed ($R^2 = 0.96–0.97$, Fig. 6c, d, Supplementary Fig. 23). Interestingly, no significant difference was found for the regression slopes between these two experimental groups, indicating these two types of stimulants independently yet equally strongly affect clot remodeling and stiffening.

To further dissect the biomechanical parameters involved in clot contraction and retraction, we performed finite element (FE) analysis of the microcot retraction process by representing the contractile platelet population using contractile 3D elements. Due to the geometrical restriction by two micropillars at both ends, microcot contraction causes volume shrinkage and the development of necking in the middle section (Fig. 6e, Supplementary movie 3), mimicking the microcot retraction process observed in the experiment. Simulated decrease in microcot volume almost linearly correlates with increased contractile stress across all force ranges (Fig. 6f), recapitulating the experimental correlation between these two measurements, as shown in Fig. 6b, d. These results suggest that clot retraction is driven by the collective contractile force of the platelet population, which may be activated by biochemical treatments and shear flows.

**Discussion**

The mechanical properties of blood clots are critical to stem bleeding upon vessel injury, and may also contribute to embolism. Despite numerous studies on platelet mechanics, little is known about the origin of tissue-level clot mechanical properties. Commonly used tissue mechanics models such as cell-laden bulk hydrogel models do not recapitulate the dynamic flow environment that is critical to clot formation and are low-throughput and time and resource intensive. We described herein a medium-throughput clotMAT system with integrated flow and mechanical measurement capacity. Using a panel of agonist and antagonist used in classical vascular injury models, we demonstrate the ability of this system to integrate the biomolecular players and shear stresses that are known to be critical in vivo. The system recapitulates the evolution of clot morphology and mechanical properties under normal and abnormal clotting conditions. Since clot mechanical properties are widely used in the clinic as indicators for blood coagulation functions, the knowledge gained in this study may help to improve the diagnosis and treatment of coagulation disorders.

Initial platelet adhesion in the clotMAT system is dependent on collagen, VWF, and the platelet receptors GpIb and GpIIbIa. In this regard, whereas blocking GpIIbIa inhibits platelet accumulation at all shear rates, GpIbα–VWF interactions
are dominant modifiers of platelet adhesion at arterial shears (Fig. 4f–j). The observation that a fibrin-platelet shell covers the collagen substrate suggests that thrombin secreted locally at the coagulation site may contribute to fibrin formation (Supplementary Figs. 5–7). Consistent with this, fibrin deposition is only observed on the collagen substrate and not other sections of the flow channel (Fig. 1). Reduction in fibrin formation upon use of PPACK, a thrombin inhibitor, resulted in unstable platelet thrombi that embolized under flow (Supplementary Fig. 15).

Nevertheless, clot contraction was measured even upon use of PPACK, although it was ~2 µN lower compared to the case of citrated PRP. Thus, contractile forces in the system are likely primarily generated by the activated platelets and transmitted via platelet interaction with the collagen substrate, presumably via receptors GpVI and VLA-237. Fibrin has an indirect role in clot retraction as it stabilizes the platelet thrombi and prevents embolization. Addition of thrombin or ADP to the flowing PRP results in higher amounts of fibrin formation and larger platelet thrombi that embolized under flow (Supplementary Fig. 15).
filled clots (Supplementary Fig. 21). Clot contraction and the impact of fibrin could not be evaluated during the course of this study, due to the massive platelet-fibrin clots that blocked the flow path. While tissue factor is absent in the current study, it is not possible to rule out the possibility that the contact pathway may also contribute to platelet deposition and clot mechanics in this system.

In clinical hematology, a major existing challenge is the diagnosis of patients whose standard laboratory test results are borderline but still have bleeding symptoms. Currently, there is no good functional assay that can be used to diagnose these patients. Thromboelastometry such as TEG and ROTEM are among the most commonly used point-of-care devices for diagnosing coagulation disorders, but it suffers from apparent limitations in diagnosing VWD and disorders of primary hemostasis^{18,19}. In the current study, we showed that clotMAT system is able to detect impaired clot formation and stiffness of VWD patient samples. Such improvement over existing clinical tests suggests that the clotMAT system may have the potential to be used to improve the diagnosis of coagulation disorders, especially for patients whose clinical phenotype cannot be determined using existing clinical tests. Preliminary results in the current study show that the clotMAT system is able to report time-dependent increases in clot mechanical properties based on the smooth ascending curves (Fig. 4b), which is qualitatively similar to the ascending curves representing clot stiffening in clinical thromboelastometry^{18,19}.

To further validate the diagnostic potential of the clotMAT system, we also tested its response to ASA since patients at risk to cardiovascular events commonly consume aspirin. The results showed that clotMAT is sensitive enough to detect the mild impairment of platelet function, clot formation and stiffness by ASA. This test, along with others performed using platelet antagonists (abciximab, anti-GpIbα) and blebbistatin, was performed using healthy blood samples collected from more than 20 healthy donors, yet the significant differences in the results were not masked by donor variation, which demonstrates the detection sensitivity of the system. Together, these data provide preliminary confirmation of the potential clinical utility of the clotMAT system. Additional comprehensive tests in a clinical setting are needed in the future to fully validate the clotMAT system. These tests may include reagents often used in TEG and ROTEM assays such as thromboplastin, heparinase and ellagic acid, different VWD/disease populations, and hyper reactive platelets from individuals suffering from cardiovascular diseases. It would be also interesting to compare the detection sensitivity of the clotMAT system directly with TEG and ROTEM. Finally, the
development of the clotMAT system was enabled by the advancement in microfabrication technology; due to its low-cost, medium-throughput and the potential for scale-up, there exists a high potential to translate this technology into an improved point-of-care system for coagulation diagnosis.

The mechanics involved in tissue remodeling has often been studied using cell-laden bulk hydrogels comprised of matrix proteins. However, due to the difficulty to incorporate flow condition and the inherent limitations in solute diffusion and experimental throughput, bulk hydrogel models are not suited for the study of clot mechanics. Previous studies using these models have shown that stiffening of the fibrin gels can be induced through either externally applied mechanical strain or myosin-mediated cellular contraction that aligns and bundles fibrin fibers and compacts matrix meshwork. However, the applicability of this strain stiffening theory under shear flow condition has not been examined. Here, through analyzing results from coagulation conditions regulated by either biochemical treatments or shear flows, we observed equally strong correlations in the microclot mechanical properties (Fig. 6a–d; Supplementary Fig. 19), indicating these two types of stimulants independently yet equally strongly affect clot remodeling and stiffening. This finding unveils the tissue remodeling/stiffening mechanism in a previously unexplored shear flow regime, and thus it significantly expands the boundary of previously established tissue remodeling theory that was based on bulk, static hydrogel models. Such integrated,
quantitative analysis of the tissue remodeling mechanism under flow condition is enabled by the combined tissue mechanics and shear flow capacity of the clotMAT system. Adding cyclic stretching function to this system may further expand its capacity to detect viscoelastic property of the clots.

Methods

Healthy donor plasma sample preparation. Human blood was obtained by venipuncture from healthy adult volunteers, following protocols approved by the UB Health Science Institutional Review Board (Buffalo, NY). Volunteers of either sex were used and studies were performed within 3 h of blood draw. Blood donors self-reported absence of coagulation defects and use of medication. Majority of the samples were prepared using sodium citrate (1:9) as anticoagulant with a few exceptions that were prepared using PPAC (70 μm, CAYman Chemicals). In some cases, PRP was recalcified by addition of 10 mM CaCl2, 2 μM Prostaglandin E1 (PGE1, CAYman Chemicals) was added when washed platelets were prepared. PRP was obtained by centrifuging blood at 180 x g for 15 min. To obtain healthy plasma or platelet-poor plasma (PPP), the remaining blood was again spun at 1200 x g for 12 min and the plasma supernatant was collected. Washed platelets were similarly obtained by further centrifuging PRP containing PGE1, at 1200 x g for 12 min. In this case, the pellet was washed once using HEPES buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 110 mM NaCl, 10 mM KCl, 1 mM MgCl2, 10 mM glucose, pH 7.4) containing 2 μM PGE1 before resuspension in HEPES buffer lacking PGE1. 1 μM BCECF (2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxyethyl Ester) (ThermoFisher Scientific) was added to PRP/washed platelets for 30 min at room temperature to stain platelets with green fluorescence.

VWF type 2A patient plasma sample preparation. VWF type 2A patient plasma from a single donor was obtained from CoaChrom Diagnostics GmbH (Austria). 0.7% agarose gel electrophoresis was used to compare VWF multimer distribution in a patient and healthy plasma17. ADAMTS13 activity was measured in terms of FRET ratio using the XS-VWF FRET substrate. Briefly, this involved addition of citrated plasma VWF to 1 μM XS-VWF FRET for 1 h at room temperature. FRET ratio was then quantified using a Synergy 4 BioTek fluorescence plate reader, based on the ratio of XS-VWF emission intensities at 541/25 nm vs. 485/20 nm. The ratio of XS-VWF emission at 420/50 nm to 480/50 nm was used as a measure of xenon-124 counting-cyberframeter-biolayer assay determined VWF concentration. Washed platelets (300,000 μM L−1) from healthy donor blood were mixed with either healthy plasma from normal donor to obtain reconstituted healthy PRP (rHealthy PRP) or VWF plasma to obtain reconstituted VWD PRP (rVWD PRP).

Microrobotic array device fabrication. The microrobotic array device was made by multilayer microlithography and soft-lithography techniques. Briefly, multiple layers of SU-8 (bottom layer for the leg section and top layer for the head section) were successively deposited on the silicon wafer (University Wafer), exposed to UV light through transparency masks printed by laser plotting (CAD/Art Services Inc.), and baked and developed according to the manufacturer’s protocols. Soft-lithography was then used to transfer the micro-patterns to polydimethylsiloxane (PDMS, Sylgard 184, DowCorning) molds made with 10:1 ratio of dimers to curing agent (Supplementary Fig. 1). The microrobotic geometry was optimized through the travel distance of the microrobots and the corresponding shear rate was 3~1500 s−1, calculated by $q = \frac{V}{Q}$, where $Q$ is the volume flow rate, $V$ and $w$ are the width and the height of the channel, respectively. In the study of the effect of shear rate on microtissue formation, the experiments at each shear rate were performed on at least three donors with at least seven samples per donor.

Microtissue contractile force measurement. HUVEC-mediated microtissue formation involves the generation of contractile force that partially remains after the removal of the HUVECs by trypsinization. Such residual contractile force of 1.88 ± 0.49 μN (153 replicates) was recorded before platelet flow. During platelet flow, bright field images of the micropillars were taken every 10 min to monitor platelet-generated contractile forces in real time. Micropillar deflection was determined by the travel distance of the micropillar head relative to the bottom of the micromanifold. The relationship used to calculate the cantilever bending theory was $F = k \delta$, where $k$ is the averaged stiffness of the two micropillars and $\delta$ is the spring constant of the microchannel (Fig. 2a and Supplementary Fig. 5). A Nikon Eclipse Ti-U inverted microscope with ×10 objective was used to image individual microtissue. During microtissue contraction, each of the micropillars displaced around 0.03 μm at 30 min after the PRP flow at 500 s−1 shear rate, so the contraction rate of the microtissue is approximately 1.7 μm min−1, which is comparable to the value reported in the previous study.

Microtissue stiffness measurement. To enable mechanical stretching, the microrobotic array device was casted on a deformable silicone substrate (silicone sheeting, 0.7% agarose gel electrophoresis, 300,000 cells per device, MI) that is mounted on a custom-made uniaxial stretching frame (Supplementary Fig. 9). Silicone substrate was stretched to 150% strain along the longitudinal direction of the microtissues to apply uniaxial tension across the entire microtissue array. Images of the microtissue and micropillars before and after stretching were recorded and used to calculate the stress and strain. Externally applied stretch caused microtissue elongation ($d_2-d_1$, and the force needed to develop such elongation was reported by microtissue contraction as $F = k (d_1 + d_2)/2$. This force was divided by the cross-sectional area of microtissue to calculate the tensile stress (Fig. 2e). The microtissue tensile strain was determined based on microtissue elongation as $\varepsilon = (d_2-d_1)/d_1$, by tracking the change in distance between two fiducial markers in the microtissues and after stretching (Supplementary Fig. 11). Subsequently, the strain was divided by the strain to calculate the elastic modulus for the microtissue. The thickness ($H$) of the microtissue for cross-sectional area calculation was determined by confocal microscopy imaging (Supplementary Fig. 12). Due to the small thickness of the assembled clotMAT device ($3-4$ mm uniform), total strain stretching across the whole device was achieved without delamination between the layers.

Platelet agonist and antagonist treatments. Many assays in the current study were performed using PRP containing various platelet agonists/antagonists. These treatments include 10 μg mL−1 Abciximab (anti-GpIIb/IIIa, Reopro, Eli Lilly), 10 μM blediblastin (Sigma), 20 μg mL−1 anti-CD42b antibody (#14-0429-80, HPI clone, ThermoFisher), and 15 μM acetylsalicylic acid (ASA, TCI America). Here, mAbs and ASA were incubated with citrated PRP for 30 min before flow and blediblastin was incubated for 5 min before flow. In some cases, 15 μM TRAP-6 (4017752, Bachem, Torrance, CA) was added to PRP prepared using PPAC just before flow. ADP (10 μM) and thrombin (5 U mL−1) were diluted in Dulbecco’s phosphate-buffered saline and infused at 500 s−1 shear rate for 10 min over microtissues previously formed under citrated PRP flow (Fig. 8b-e). In some cases, ADP (5 μM) and thrombin (2 U mL−1) were directly added to citrate PRP before flow to study their effects on clot formation (Supplementary Fig. 21). For all the agonist and antagonist treatments, at least three donors with at least seven samples per donor were measured.

Platelet activation studies. PRP was isolated from fresh human blood using either 70 μM PPAC or 3.8% sodium citrate (1:9) as anticoagulant. In some cases, PRP was recalcified by addition of 10 mM CaCl2. 50 μL PRP was incubated with a mixture of anti-human CD41/CD61 activation-specific mAb PAC-1 (Alexa647 labeled, #362805, Biolegend, San Diego, CA) and PE-labeled anti-CD62P mAb (153 replicates) was recorded before platelet flow. During platelet flow, bright field images of the micropillars were taken every 10 min to monitor platelet-generated contractile forces in real time. Micropillar deflection was determined by the travel distance of the micropillar head relative to the bottom of the microchannel. The relationship used to calculate the cantilever bending theory was $F = k \delta$, where $k$ is the averaged stiffness of the two micropillars and $\delta$ is the spring constant of the microchannel (Fig. 2a and Supplementary Fig. 5). A Nikon Eclipse Ti-U inverted microscope with ×10 objective was used to image individual microtissue. During microtissue contraction, each of the micropillars displaced around 0.03 μm at 30 min after the PRP flow at 500 s−1 shear rate, so the contraction rate of the microtissue is approximately 1.7 μm min−1, which is comparable to the value reported in the previous study.

Fabrication and integration of the microfluidic channel. The PDMS microchannels measuring 1189 μm in width and 498 μm in height were fabricated using standard photolithography and soft-lithography methods. The microchannels were aligned with individual rows of trypanized microtissues under stereomicroscope, and the PDMS slab containing microchannels was bond with the PDMS substrate containing microtissue array through custom clamps (Supplementary Fig. 4). The platelet flow was created through syringe pump (NE-1000, New Era Pump System) withdrawn at the outlet of the micromanifold. Both the inlet and outlet of the microchannel were connected to the flow system through 18G needles. Flow rate was set between 0.01 and 4.5 mL min−1 and the corresponding shear rate was 3~1500 s−1, calculated by $q = \frac{V}{Q}$, where $Q$ is the volume flow rate, $V$ and $w$ are the width and the height of the channel, respectively. In the study of the effect of shear rate on microtissue formation, the experiments at each shear rate were performed on at least three donors with at least seven samples per donor.
the sample was diluted 200-fold at various times and analyzed using a BD Fortessa X-20 flow cytometer (Supplementary Fig. 16).

Fibrin formation assay under static condition. Alexa fluor 594-conjugated fibrinogen (Fb-594) was prepared by mixing 10 mg/mL-1 fibrinogen (#9001-32-5, Sigma) with 20-fold molar excess of Alexa fluor 594 (NHS ester, #1101-1, Fluoroprobe, Scottsdale, AZ) in PBS (pH = 8.0) for 1 h at room temperature. Following quenching of the reaction using 100 µL 1 M Tris-HCl (pH = 8.0) for 10 min, labeled protein was desalted into PBS using a Zeba Spin Column according to the manufacturer’s instructions (7K MWCO, #89882, ThermoFisher).

To assay fibrin formation, 0.125 mg mL−1 Fb-594 was added to PRP (20 µL volume). Following this, various stimuli were added: 15 µM TRAP-6, 5 U mL−1 Thrombin, 20 µM ADP or 5 µg mL−1 collagen. Such assays were performed using citrated PRP, recalciﬁed citrated PRP and PPACK anticoagulated PRP under static condition. Following 10 min, 150 µL PBS containing 5 mM EDTA was added to stop the reaction. Fibrin clot was then immediately pelleted using centrifugation at 14,000 × g for 10 min, and Alexa 594 fluorescence in supernatant was measured using a plate reader in order to determine % Fb-594 incorporated into fibrin clot (Supplementary Fig. 17). Calibration curve was made by serial dilution of Fb-594.

Shear-induced platelet activation (SIPAct). Two microliters citrated healthy PRP was diluted 40-fold into either healthy human plasma (PPP) or VWD type 2A plasma. The mixture was sheared in a cone-plate viscometer at 9600 ± 1 r.p.m. Samples withdrawn at various times were incubated with Annexin-V FITC for 5 min at 37 °C in HEPES buffer containing 5 mM CaCl₂ prior to ﬂow cytometry analysis. % Platelet activation is a measure of the fraction of platelets binding greater than baseline levels of Annexin-V (Supplementary Fig. 22).

Immunofluorescence staining and imaging. Microtissues were ﬁxed using 4% (v/v) paraformaldehyde (PFA) (EMS, Hatﬁeld, PA) overnight and labeled with Alexa Fluor v) paraformaldehyde (PFA) (EMS, Hatﬁeld, PA) overnight and labeled with Alexa Fluor 594 (NHS ester, #1101-1, Fluoroprobe, Scottsdale, AZ) in PBS (pH 7.4) for 1 h at room temperature. Following quenching of the reaction using 100 µL 1 M Tris-HCl (pH 7.4) for 10 min, labeled protein was desalted into PBS using a Zeba Spin Column according to the manufacturer’s instructions (7K MWCO, #89882, ThermoFisher).

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Author contributions
R.Z. and S.N. conceived the idea; Z.C., J.L., C.Z., I.H., X.Y., L.M., E.M., M.A. and R.Z. performed the experiments and analyzed the data; Z.C., S.J., S.N. and R.Z. wrote the manuscript.

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