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REVIEW

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Microfluidic thrombosis analysis system: possibilities and limitations

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Abstract

Thrombosis is a double-edged sword. Normal thrombus formation within injured blood vessel is an important natural defensive mechanism to prevent excessive bleeding, whereas abnormal thrombus formation leads to critical disease such as stroke or myocardial infarction. One of keys in the pathophysiology mechanism involved in the thrombus formation is acute hemodynamic changes within the vessel lumen, which has been investigated mostly in pre-clinical and clinical studies. However, studies involving animal or human subjects are frequently limited by technical difficulties and requirement of substantial blood volume. Microfluidic systems have emerged as a valuable tool owing to their inherent advantages including minimal sample requirements and rapid analysis capabilities. In this mini review, we present a summary of microfluidic systems designed for thrombosis analysis, encompassing fabrication processes, design, and analysis methods. We also discuss both the potentials and limitations of microfluidic platform for the analysis of thrombus mechanisms.

Keywords Thrombosis, Microfluidics, Wall shear rate, Recirculation zone, Soft lithography, Image analysis

Introduction

Thrombus formation is a natural response to external stimuli or injury and also pathophysiological phenomenon causing critical thromboembolic diseases [1, 2]. It is a complex process driven by biochemical interactions and hemodynamic changes among blood cells, plasma proteins, and vessel wall [3]. Extensive research has been conducted to identify the underlying causes of abnormal thrombus formation [3]–[7]. Among these hemodynamic factors, shear rate is considered as a major contributor to thrombus formation [8]. The magnitude of the shear rate varies greatly with the diameter of the blood vessel, leading to variations in the mechanisms of thrombus formation (Fig. 1).

Thrombus formation at low shear rates is described by Virchow's triad. Virchow's triad is classic thrombus formation mechanism comprising three key components: endothelial injury, hypercoagulability, and blood flow stasis [1]. Stagnant flow is commonly observed in conditions such as aneurysms and venous valve [9]–[11]. With the reduced shear rates, red blood cells and platelets form aggregations through the FasL/FasR pathway [12] and fibrinogen protein [10]. It forms 'red thrombus' due to the presence of a high concentration of red blood cells.

Unlike the red thrombus, 'white thrombus' is common in thrombus in artery which is characterized by relatively fast and complex flow patterns [13]. It primarily consists of activated platelets that bind to von Willebrand factor (vWF) rather than fibrinogen [13]. Meanwhile, fibrinogen is converted to fibrin through the action of thrombin. Fibrin molecules polymerize and form a threadlike structure that effectively stabilizes the thrombus [14].

Understanding these thrombus formation mechanisms could be used to develop drugs to treat thrombosis. From this perspective, microfluidic systems offer advantages and have become a valuable tool for dissecting the

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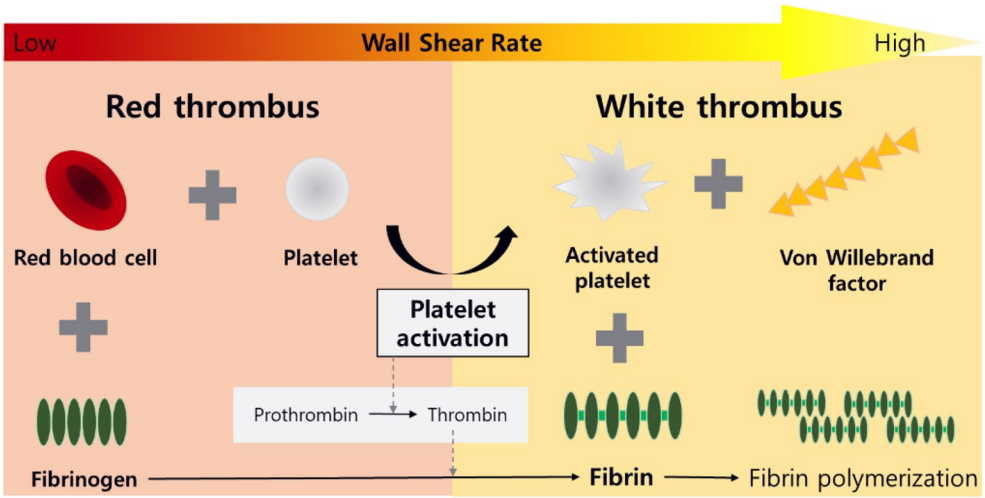

Fig. 1 The mechanism of thrombus formation based on varying shear rates. Under low shear rates, thrombus primarily consists of red blood cells and fibrinogen, displaying red color. Elevated shear rates trigger platelet activation and lead to the aggregation of activated platelets with vWF and fibrin, which results in the formation of white thrombus

mechanisms behind thrombus formation [15]. Importantly, the use of microfluidic chips allows a minimal amount of precious blood to be utilized. It reduces the burden of blood donors and shortens the analysis time [16]. Furthermore, microscale systems have the potential to enable point-of-care (POC) diagnostics for emergency situations [17]. Transitioning from POC potential to real-world application involves several important considerations. In this mini-review, we present representative research examples of microfluidic systems for thrombus analysis. These examples are summarized in Table 1.

Fabrication

2D geometry

The conventional approach for producing microfluidic chips is soft lithography [8, 18]–[20]. It necessitates the creation of a master mold through a photolithography procedure. Subsequently, a flexible material, like polydimethylsiloxane (PDMS), is poured into the master mold and allowed to cure. Once fully cured, it is carefully detached from the master mold to yield a replica bearing the desired pattern. Fabrication techniques employing these replicas include replica molding, micro-contact printing, and micro-transfer molding [19].

Replica molding generates microchannels by combining a replica with a substrate. E. Westein et al. [18] employed replica molding to create microchannels resembling the shape of atherosclerotic plaques, as shown in Fig. 2a. In this method, PDMS was poured onto a master mold composed of SU-8 material and left to cure overnight. The completely cured PDMS was separated from the master mold. Subsequently, it was affixed to a surface-treated glass coverslip using plasma bonding that effectively seals it in place.

Next, the following two methods employ replicas as tools in the fabrication process. In microcontact printing, a replica functions as a stamp for transferring molecular ink onto a substrate. This technique uses self-assembled monolayers (SAMs) to create precise molecular-level patterns on the substrate surface [20]. Meanwhile, micro-transfer molding utilizes a replica as a mold to contain other polymer solutions. The replica is attached to the substrate and subsequently removed once the internal polymer is solidified. The micro-transfer molding minimizes potential damage to the master mold [19].

Microchannels fabricated by soft lithography has several advantages. First, master molds featuring microscale patterns can be efficiently produced via the photolithography process. These master molds are reusable, enabling the repetitive production of replicas and thus reducing costs. However, it's worth noting that most master molds typically have square cross-sections and differ in shape from human blood vessels. In addition, the creation of a master mold precedes the soft lithography process and has the limitation of simple two-dimensional structures to facilitate the easy removal of replicas. While three-dimensional microchannels can be achieved by stacking multiple replicas, aligning these replicas is challenging.

3D geometry

Various processing techniques have been developed to overcome the limitations associated with soft lithography [21]–[26]. T. Q. Nguyen et al. employed thermal expansion of air to produce microchannels with cross-sections

Table 1 Summary of thrombosis research using microfluidic chips

Control variable	Magnitude of SR [s^{-1}]	Fabrication	Sample	Analysis method	Refs
Hemodynamics					
Low SR	< 5000	Photo- & Soft-lithography	Human blood	Fluorescence microscopy Image processing	[43, 46]
		3D printing	Human blood	Culture of ECs Fluorescence microscopy Image processing	[25]
High SR	> = 5000	Photo- & soft-lithography	Human blood	Image processing	[52]
		Micromilling & soft-lithography	Porcine blood	Light transmission detection system Image processing	[5, 33]
Gradient of SR	500–20000	Photo- & Soft-Lithography	Human blood	Fluorescence microscopy Image processing SEM Micro-PIV	[3, 6, 7, 35]
Degree of stenosis	600–29859	Photo- & Soft- Lithography	Human blood	Culture of ECs Fluorescence microscopy Image processing Staining	[18, 22, 32, 44]
Number of stenosis	> = 12,000	Photo- & Soft- Lithography	Human blood	Image processing	[4]
Biochemistry					
Kinds of thrombolytic agents	3500	Capillary tube	Porcine blood	Fluorescence microscopy Image processing	[31]
Dose of antiplatelet therapy	500–10000	Micromilling & Soft-lithography	Human blood	Impedance aggregometry	[34]

SR shear rate, *ECs* endothelial cells, *PIV* particle image velocimetry, *SEM* scanning electron microscope

resembling blood vessels [21, 22]. PDMS replicas with rectangular cross-sections were placed on partially cured PDMS and sealed by themselves. When exposed to a high-temperature environment, the air trapped within the replica expanded while the PDMS fully cured. This interplay between air expansion and PDMS curing formed semicircular cross-sectional channels. Then semicircular channels were bonded to another partially cured PDMS layer and thermally expanded to create channels with circular cross-sections. There was no need of complex alignment procedures.

Furthermore, a method for producing microchannels with circular cross-sections using fibers was developed [23, 24]. H. Hong et al. aimed to replicate blood flow characteristics in stenotic vessels [23]. They achieved this by sanding the middle section of an optical fiber to create a stenosis section. PDMS was then poured over the prepared optical fiber and fully cured. Upon removal of the optical fiber, a microchannel with a circular cross-section and a stenotic region was generated. A limitation was that it could only produce straight channels.

3D printing offers a convenient means to fabricate complex three-dimensional channels. P. F. Costa et al. compared thrombus formation between healthy individuals and patients with stenosis using 3D printed channels [25]. Vascular geometry information was sourced from digital imaging and communications in medicine (DICOM) files and then used to convert 2D images of vascular tissues into 3D models. After casting PDMS into the 3D-printed mold, the mold was removed, leaving behind the completed microchannel (Fig. 2b). One notable advantage of 3D printing technology is its ability to complete the manufacturing process in one step by providing geometric information [19]. However, it faces challenges when attempting to produce extremely fine-sized channels due to inherent instrument limitations, and the resulting surface is generally much rougher compared to soft lithography.

Surface preparation

In addition to manufacturing methods, it is important to pay close attention to the surface of the microchannel that directly contacts blood. The environment of the surface of the channel is different from human blood vessels, which can influence thrombus formation and affect experimental outcomes [27]. To mitigate the unintentional formation of thrombi and ensure the reliability of experiments, researchers often explore surface modifications such as coating of anticoagulant including heparin [28]. Conversely, in specific area such as stenosis, it is essential to mimic a pathological condition by coating the area of interest with thrombotic materials. The choice

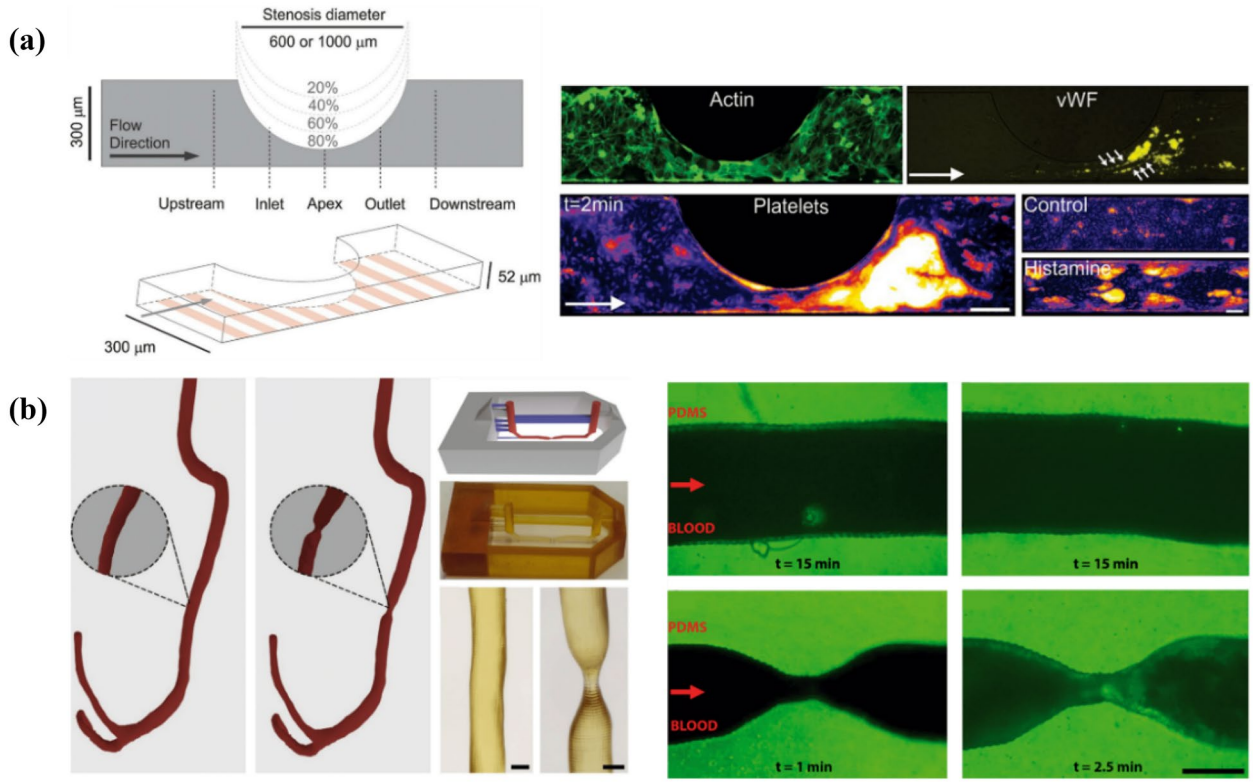

Fig. 2 Microfluidic chips utilized in thrombosis research. **a** Microchannel created using soft lithography. Thrombus formation by perfusing blood into channels where endothelial cells were cultured. Adapted from Westein et al. PNAS 2013;110(4):1357–1362 [18]. **b** Molds having in-vivo shapes were manufactured using an SLA-based 3D printer. Thrombus formation between healthy human vessels and patients with stenosis was compared by perfusing blood into PDMS channels. Reproduced from Costa et al. Lab Chip 2017;17:2785–2792 [25]

of thrombotic materials varies depending on the experimental objectives: collagen and vWF for atherosclerosis, tissue factor for vascular injury, and fibrinogen at low shear rates [29].

Design

Laminar flow

The channels in microfluidic chips form laminar flows characterized by low Reynolds numbers. Similarly, natural blood flow in human vessels typically exhibits laminar characteristics. Shear rates play a pivotal role in platelet activation [30]. The magnitude of shear rates is determined by the Hagen-Poiseuille equation. Therefore, many previous studies introduced stenotic regions within microfluidic chips to achieve higher shear rates [22, 31, 32].

M. Li and colleagues devised four microchannels, featuring stenosis sections to achieve a range of shear rates (500, 1500, 4000, 10,000 s^{-1}) (Fig. 3) [33]. Each tube served as a resistance element within the interconnected microchannel. The shear rate threshold for thrombus formation was found to exceed 4000 s^{-1}. Additionally, these microchannels were employed for a comparative study evaluating the effectiveness of antiplatelet drugs [34].

Another research team, led by A. Fouras and S. P. Jackson, focused on the investigating shear rate gradients [35]. They designed a microchannel consisting of three distinct regions: an acceleration region (1800 ~ 20,000 s^{-1}), a peak region (20,000 s^{-1}), and a deceleration region (20,000 ~ 200 s^{-1}). Interestingly, thrombus formation was primarily observed in regions characterized by decreased shear rates. W. S. Nesbitt et al. emphasized the role of platelet membrane tethers [35]. Platelet membrane tethers are tail-shaped extensions originating from the platelet membrane and involved in platelet adhesion [36, 37]. Tethers undergo activation in the acceleration section and subsequently become more firmly coupled and stabilized in the deceleration section [35].

Complex flow

As blood vessel system is branching hierarchical structure, flow pattern in the human blood vessel is not always laminar flow and frequently shows turbulence or complex pattern. One notable example is the recirculation zone. It is the vortex area where activated platelets and

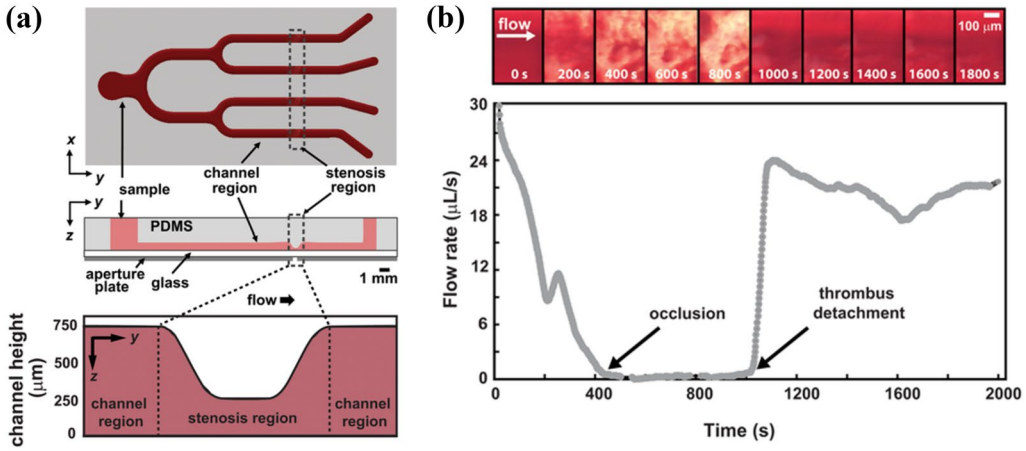

Fig. 3 A device designed for observing thrombus formation under high shear rates. **a** Design with a focus on achieving high shear rates in the stenosis section. **b** The process of thrombus formation and detachment over time. Reproduced from Li et al. PLoS ONE 2014;9(1):e82493 [34]

procoagulant proteins can accumulate. Recirculation zones prolong the residence time of blood and serve as the environment for cell aggregation. Recirculation zones can manifest not only behind obstructions [38] but also at vascular branches and within aneurysms [39]–[41].

Z. Schofield and colleagues investigated the effect of valve stiffness on thrombus formation in deep vein thrombosis (DVT) [42]. DVT is a condition characterized by the formation of blood clots in the deep veins of lower legs. It is well-known that the stiffness of venous valves tends to increase with age. The valve stiffness correlates with the recirculation area around the valve. The researchers manipulated the amount of photo-initiator (PI) during the chip fabrication process, resulting in the production of valves with varying degrees of rigidity and recirculation areas of different sizes. Notably, the size of the recirculation area directly influenced the retention and attachment of particles (Fig. 4).

However, the microenvironment of experimental particle aggregation using polystyrene particles and whole blood differs from the microenvironment of the actual thrombus formation. Incorporating complex flows including recirculation regions, into microchannels is challenging. As a result, many studies have been conducted based on simulation analysis rather than physical experiments. Nevertheless, there remains a need for the thrombus formation under complex flow conditions as most thrombosis occurs at the site of vessels with complex flow.

Analysis methods

Dynamic condition

The analysis of thrombus formation under dynamic conditions relies on image processing techniques, particularly the observation of specific cell behaviors using fluorescent materials. Fluorescent agents bind to biomarkers such as platelets and fibrin, providing visual insights into the thrombus formation process. J. Berry et al. compared thrombus formation in single channels with that in pressure relief channels (Fig. 5). They observed the adhesion of platelets, leukocytes, and fibrin, each tagged with different fluorescent colors [43]. This method provides clear and intuitive information about the cellular components involved in thrombus formation. While it is possible to employ two or more fluorescent markers within a single experiment, real-time monitoring was constrained by the choice of markers with similar wavelengths. Detecting cells using multiple colors necessitates post-processing for verification.

However, the use of fluorescent materials necessitates specialized equipment such as a fluorescence microscope or confocal microscope and may affect thrombus formation process. J.-S. Choi et al. achieved visualization and quantification of thrombus without fluorescent materials. They utilized a simple experimental setup and image processing techniques [44]. Whole blood was perfused through microchannels with circular stenotic cross-sections. The process of thrombus formation within the channels was recorded using optical microscopy. Over time, images were extracted from the video and adjusted for contrast and brightness to enhance thrombus visibility. The image analysis revealed that thrombus formation was followed by a sequence of events: adhesion to the channel wall, aggregation, and occlusion. Additionally, not only the thrombosis but also the embolization could be captured.

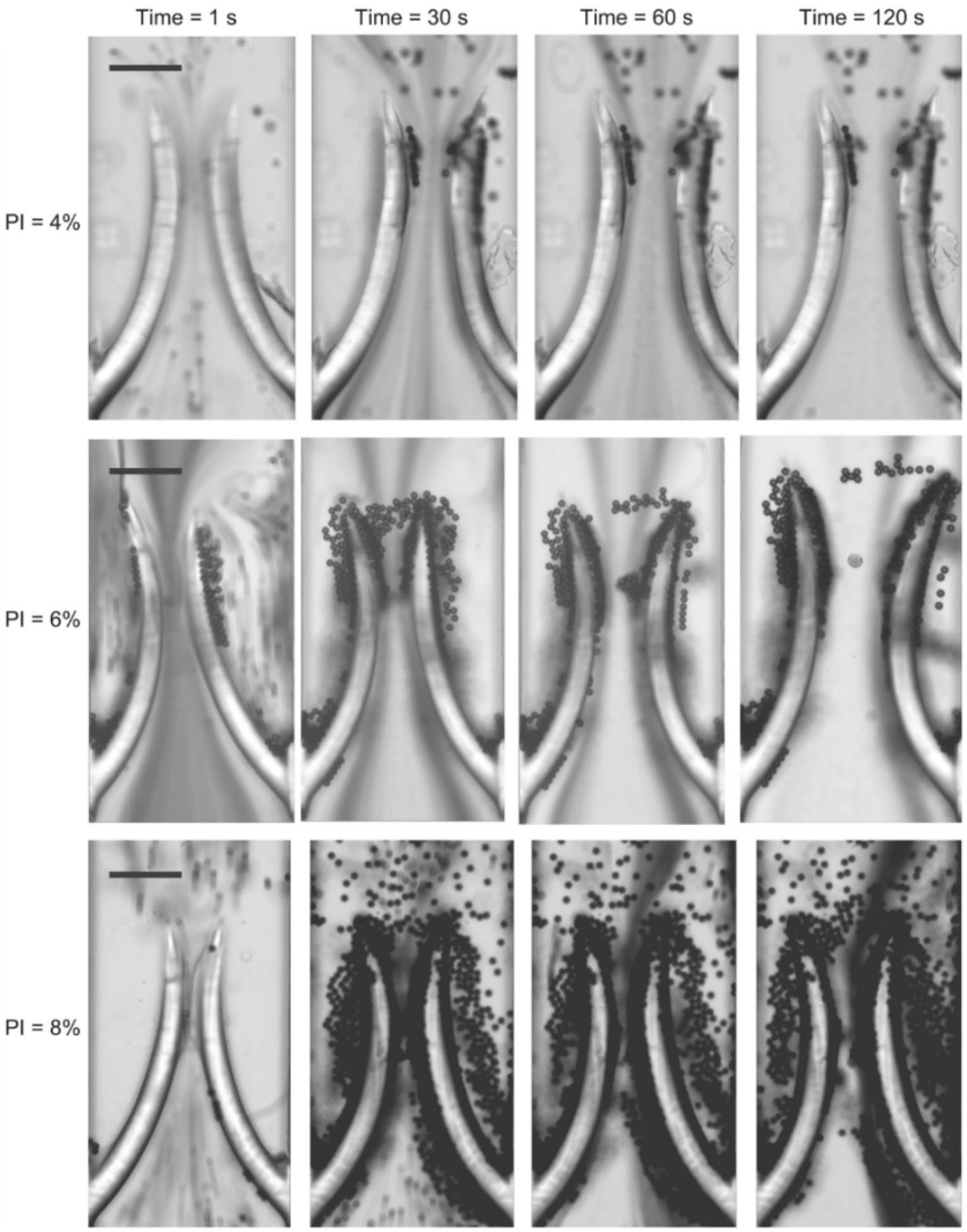

Fig. 4 The accumulation of particles in the recirculation zone due to the presence of a structure resembling a vein valve over time. Valve structures with different photo-initiator (PI) mixing ratios (4%, 6%, and 8%) exhibited different stiffnesses. The degree of particle aggregation and valve stiffness was analyzed by measuring polystyrene particles in the recirculation zone around the valve. Adapted from Schofield et al. Commun Mater 2020;1(65) [42]

Static condition

After the blood experiments, thrombi within the microchannel can be examined under static conditions. Typically, scanning electron microscopy (SEM) [45, 46] and staining methods [47, 48] have been employed to visually study the thrombus structure. D. N. Ku group recently utilized the aforementioned methods to analyze the structure of thrombus formed under high shear rates (Fig. 6) [49].

Morphology

SEM was used to examine both the morphology of the thrombus and the density of platelets. Thrombus formed under maximum shear rates (exceeding 10,000 s^{-1} within

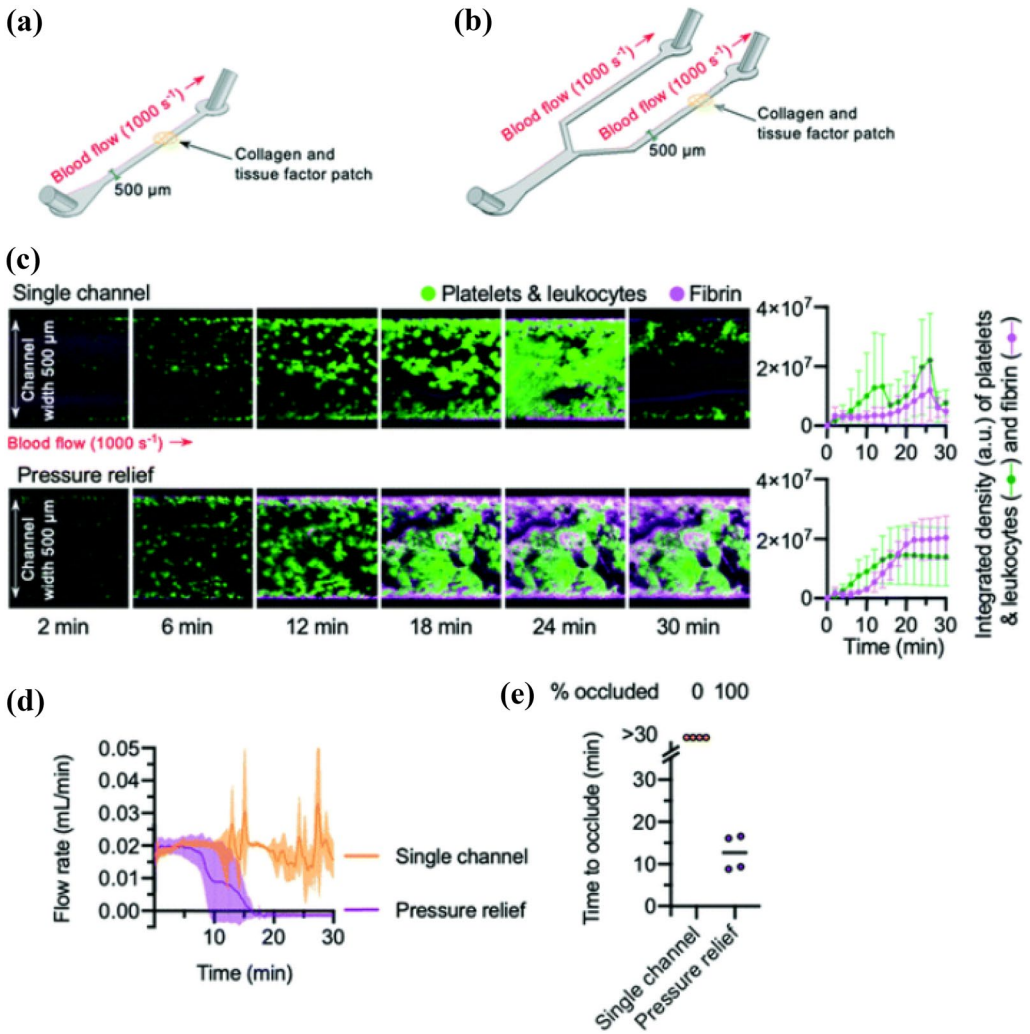

Fig. 5 Analysis of platelets, white blood cells, and fibrin using fluorescent materials [43]. Platelets and white blood cells were identified using the lipid dye $DiOC_6$, while fibrin was detected using fibrinogen Aleza-546 conjugate. A comparison was made between **a** a single channel and **b** a pressure relief channel. **c**–**e** The beneficial impact of the pressure relief design on promoting stable thrombus formation was reported. Adapted from Berry et al. Lab Chip 2021;21:4104–4117 [43]

the stenosis section) was initially preserved using a 10% formalin solution. After fixation, the thrombus was submerged in an ethanol solution and subsequently rinsed with distilled water. The prepared sample was left to air-dry overnight and then coated with a layer of Au via sputtering to facilitate SEM observation. Upstream and downstream of the stenosis section, non-activated spherical platelets were observed (Fig. 6b). In contrast, the middle of the stenosis displayed malformed platelets, which showed the effects of high shear rates.

Histology

Histological analysis was employed to explore the composition of the thrombus. Initially, the thrombus was preserved by fixation in formalin and subsequently embedded in paraffin. The paraffin-embedded thrombus was sliced to a thickness of 5 μm, and then paraffin was carefully removed. To distinguish various components, including platelets, fibrin, red blood cells, and white blood cells, Carstairs' staining method was used (Fig. 6c). Within the central region of the stenosis area, 80% of the composition was comprised of platelets, with 5% being vWF and fibrin. Dense platelet aggregation was observed near the channel walls. Upstream and downstream of the stenosis section, fibrin, vWF, and some inactive platelets were identified.

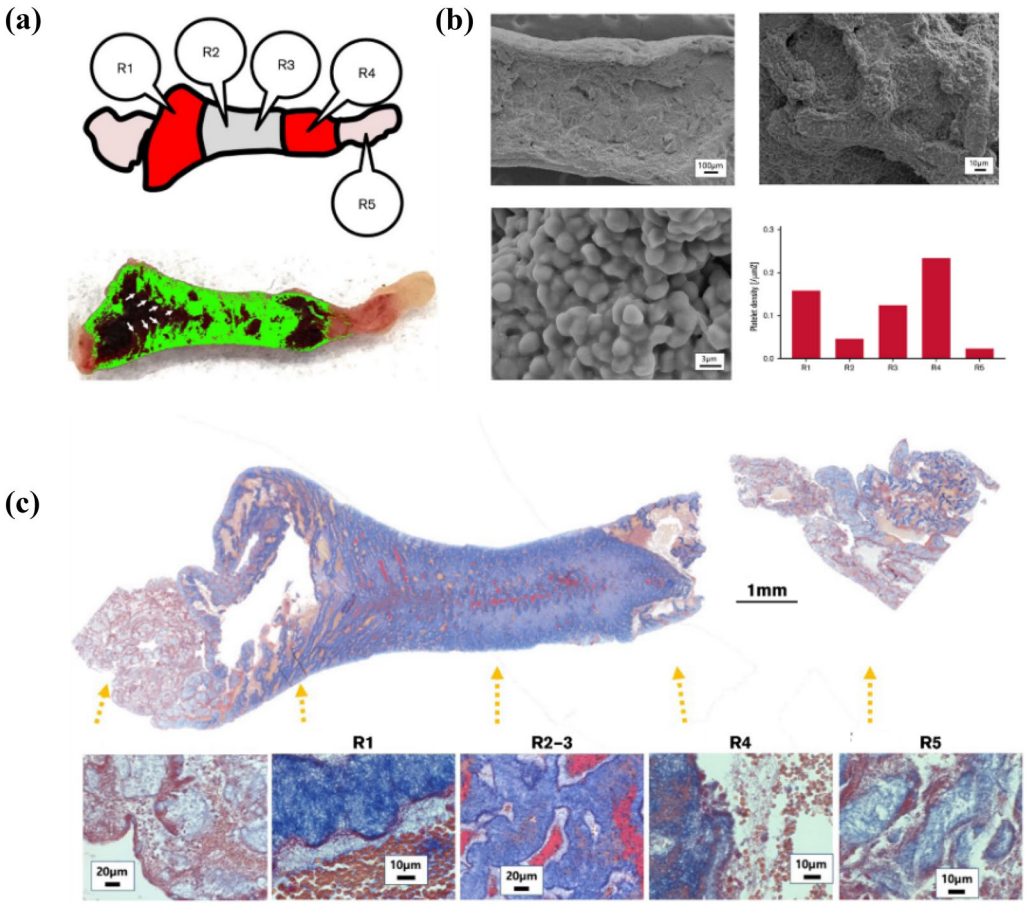

Fig. 6 Thrombus analyzed under static conditions. **a** Thrombus segments within the thrombus for static analysis. **b** SEM images of thrombus formed in the microchannel. **c** Distribution of blood cells and proteins verified using Carstairs' staining method. Adapted from Kim et al. Blood Adv 2022;6(9):2872–2883 [49]

Future perspectives

Complex flow implementation

Microchannels exhibit low Reynolds number flow due to their small caliber. In contrast, human blood vessels often feature complex flows characterized by high Reynolds numbers. Achieving high Reynolds numbers within microchannels demands fast flow rates, which can impact channel design and durability. In addition, replicating intricate flow patterns, such as recirculation zones, within microchannels can be challenging [29, 50].

Mechanical movements

While blood vessels can contract and expand in response to hemodynamic changes, the geometric shape of a microfluidic chip allows minimal deformation despite being constructed from flexible materials. To replicate the dynamic movements of natural vessels within a microfluidic system, supplementary actuators are required to be incorporated into the setup [51].

Biochemical interactions

In-vitro analysis systems aim to replicate in-vivo environment while providing cost-effective and efficient research tools. However, processes in sample preparation may alter or influence the condition of blood. Typically, blood is treated with anticoagulants and preserved. Just before experiments, coagulation ability is restored by adding solutions antagonizing the effect of anticoagulants. Although these chemical treatments are de facto standard in experiments, their potential influence on the thrombus formation should not be overlooked. Additionally, it's important to note that not all biochemical reactions occurring in natural blood vessels can be replicated within microchannels. In-vitro analysis systems can offer a controlled environment tailored to specific research objectives.

Monitoring system

In previous studies, thrombus formation was observed in real-time under a microscope [5, 33, 44]. However, this

observation method mainly enables the assessment of thrombus growth in terms of area rather than volume. Furthermore, continuously monitoring of single area for an extended duration is inefficient. Despite the rapid occurrence of thrombus formation due to platelet activation [33], it is advisable to consider adopting a multi-region monitoring system to enhance research efficiency.

Conclusion

This review discussed the potential of investigating thrombi using microfluidic systems encompassing chip fabrication methods, design considerations, and analytical approaches in detail. Microfluidic analysis systems offer numerous advantages, with a key feature being their ability to provide rapid and precise diagnoses with minimal sample volumes and offer real time observation. These advancements in thrombosis research hold promise for the development of treatments for thrombosis, including antiplatelet agents and blood analysis.

Abbreviations

vWF	Von Willebrand factor
POC	Point-of-care
PDMS	Polydimethylsiloxane
SAMs	Self-assembled monolayers
DICOM	Digital imaging and communications in medicine
DVT	Deep vein thrombosis
PI	Photo-initiator
SEM	Scanning electron microscopy

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Availability of data and materials

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Declarations

Competing interests

Not applicable.

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