Mini-Review

Bioengineered *in vitro* models of thrombosis: methods and techniques

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Abstract: Thrombosis is a prevailing vascular disorder that has been historically studied *in vivo* with conventional animal models. Here we review recent advances in methods and techniques that allow for engineering of biomimetic *in vitro* models of thrombosis, usually combined with microfluidic devices, termed thrombosis-on-a-chip systems, to reproduce such vascular pathology outside living organisms. These human cell-based thrombosis-on-a-chip platforms recapitulate the important characteristics of native thrombosis in terms of vascular structures, extracellular matrix properties, cellular composition, and pathophysiology, making them enabling *in vitro* models to study this important class of vascular disorders as well as to develop personalized treatment regimens.

Keywords: Interventional radiology; thrombosis; thrombosis-on-a-chip; soft lithography; bioprinting; blood vessels

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Introduction

Thrombosis is a prevailing vascular disorder that may readily occur in both arteries and veins (1-4). Thrombosis is often the result of vascular injuries, featured by obstruction, to different degrees, of vascular lumens by coagulated blood and thus disturbance/cessation of blood flows at the site surrounding the thrombus (5,6). In healthy vessels, the endothelium functions to prevent coagulation, through secretion of anti-thrombotic agents such as nitric oxide and expression of binding sites for similar molecules such as antithrombin (5,7,8). Therefore, injuries of the endothelium usually lead to the activation of primary homeostasis (platelet binding and aggregation) and the coagulation cascade (fibrin formation and crosslinking) (*Figure 1A*) (5,7,8,10). While acute thrombosis may be resolved via administration of thrombolytic agents such as tissue plasminogen activator (tPA) that degrade fibrin (11-14), this is not always effective. The invasion and propagation of fibroblasts and smooth muscle cells from the site of endothelial injury into the mass of thrombus usually result in its fibrosis and matrix organization, turning the thrombus to a permanent clot that cannot be resolved (*Figure 1B*) (15-20). Subsequently, the vessel wall stiffens and blood pressure builds up (21,22), further developing post-thrombotic syndrome, clinically manifesting as symptoms including edema, pain, and ulceration, among others (15,18).

While animals have been historically used as models to study thrombosis (23-25), these *in vivo* models typically differ from the human system, leading to biased understanding and inaccurate predictions of treatment effects in humans. In addition, imaging and characterizing thrombosis in animals are cumbersome, and they cannot achieve high throughput that is needed in cases such as screening of therapeutics. Here, we discuss some recent advances in methods and techniques used for generating *in vitro* biomimetic models of human thrombosis, termed thrombosis-on-a-chip systems, which to a large extent

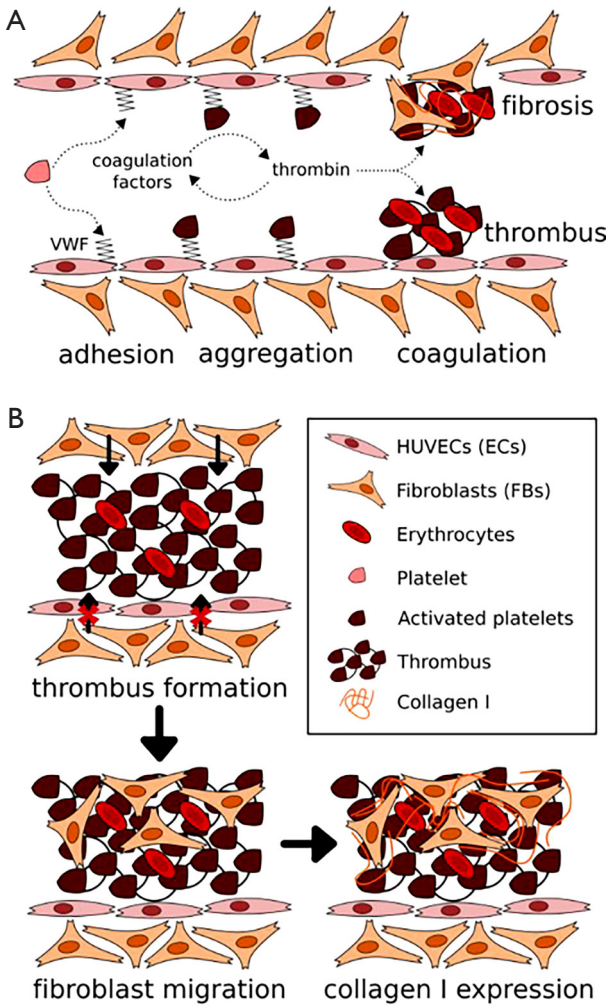

Figure 1 Thrombosis and vascular injury. (A) Mechanism of thrombus formation and development. After the endothelium damage, platelets adhere to the endothelial layer via vWF. This leads to the activation and finally aggregation of platelets. Simultaneously, coagulation cascade results in cleavage of fibrinogen to fibrin through thrombin, to eventually form a thrombus. (B) Formation of fibrotic thrombus through fibroblast migration into the blood clot from the surrounding tissue via the damaged endothelium. Collagen type I is then expressed by the invading fibroblasts, leading to fibrosis. Adapted with permission from Ref. (9), copyright 2016 Royal Society of Chemistry. vWF, von Willebrand factor.

recapitulate the important pathophysiology of their *in vivo* counterparts in terms of vascular structures, extracellular matrix properties, and cellular composition. These miniaturized, usually transparent devices also allow for convenient analyses at much increased throughputs than animals. We finally conclude with future perspectives. It should be noted that, only hydrogel-based thrombosis-on-a-chip models will be illustrated in the current Review, as they typically present better biomimetic features than those fabricated with silicone elastomers and plastics.

Thrombosis-on-a-chip models generated using soft lithography

Soft lithography is a long-established technique for patterning a variety of (bio)materials at microscales (26). It is generally conducted through a replica molding process, i.e., a master mold with a desired pattern is first fabricated, on top of which the secondary material is cast and crosslinked; this secondary material is then detached from the master mold to achieve the replication of the pattern in the reverse mode. As such, the interconnected microfluidic channels inside a bulk material can be readily produced to mimic the perfusable vascular structure (27,28). Soft lithography is easy to operate, capable of high-resolution patterning, and highly reproducible, but is sometimes limited by the reliance on the need for the master molds with pre-designed primary patterns.

In a prominent example, Zheng and colleagues utilized a vascular network generated with soft lithography to study angiogenesis and thrombosis (29). In their procedure, a polydimethylsiloxane (PDMS) stamp containing a surface pattern of interconnected ridges was cast with a bath of collagen solution; upon gelation, the PDMS stamp was removed to expose the patterned grooves at the bottom of the collagen hydrogel, followed by attachment to another layer of flat collagen at the bottom to form a closed vascular structure within the collagen matrix (*Figure 2A*). Subsequently, endothelial cells were seeded into this hollow vascular pattern, leading to the formation of a monolayer of endothelium on the interior surface of the microchannels (*Figure 2A,B*).

With this perfusable vascular device, the authors were able to further study the interactions of whole blood and the endothelium. As expected, under normal conditions, the vast majority of blood cells flowed past the endothelial surfaces without noticeable adherence, and only a small amount of platelets were observed to roll along the endothelium during the blood flow (*Figure 2C*). On the contrary, when the vascular chip was primed with phorbol-12-myristate-13-acetate (PMA), a known secretagogue for von Willebrand factor (vWF), the platelets started to significantly aggregate and adhere to the endothelial surface

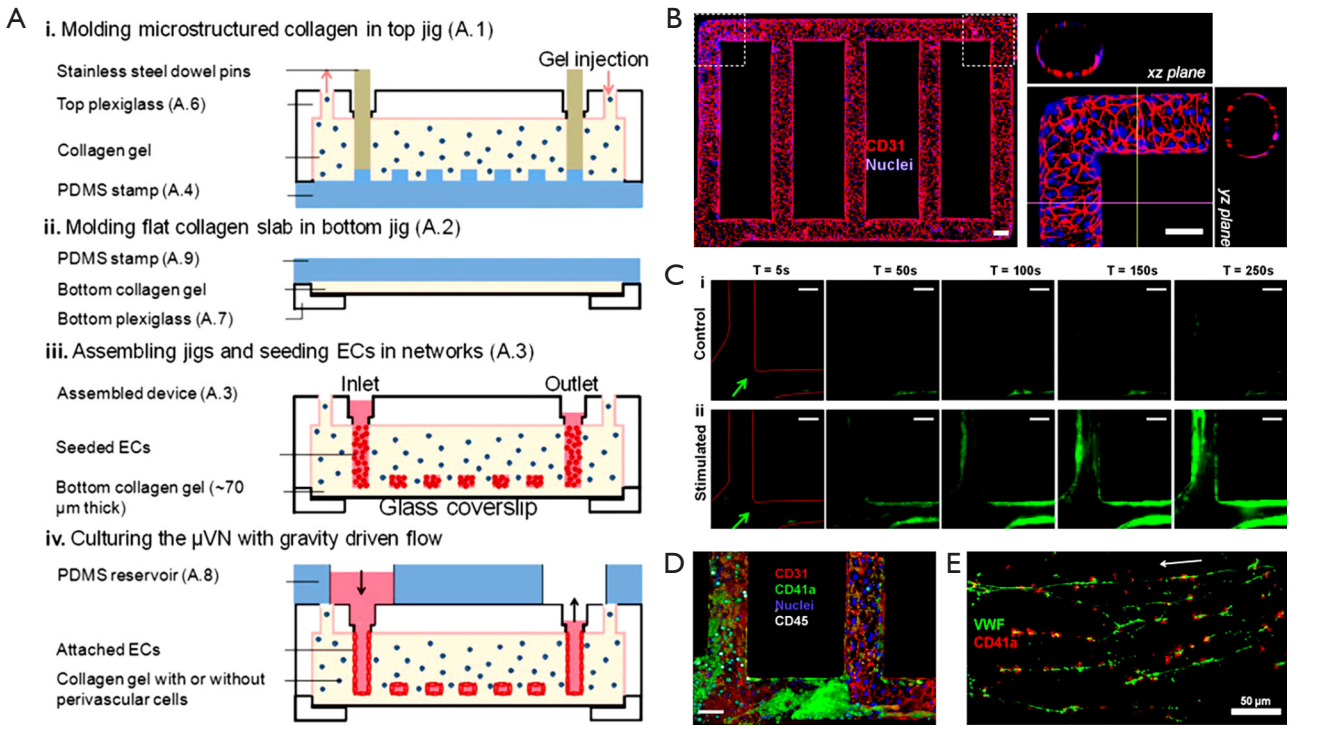

Figure 2 Thrombosis-on-a-chip model generated using soft lithography. (A) Schematics of the soft lithography fabrication procedures of the vascular chip: (i) molding the microstructured collagen in top jig; (ii) molding flab collagen slab in bottom jig; (iii) assembling the jigs mechanically and seeding cells through the inlet and outlet reservoirs; and (iv) culturing the microvessel network with gravity-driven flow. (B) Confocal projection view of endothelialized microfluidic vessels (overall network, left) and orthogonal views of the corner. Scale bars: 100 μm. (C) Time sequences of whole blood perfusion through a vascular chip, either quiescent (control vessels, i) or (ii) stimulated, at a flow rate of 10 μL min^{-1} at time points of 5, 50, 100, 150, and 250 s after initiation of perfusion. The platelets are in green, labeled for CD41a to platelet-specific glycoprotein IIb (integrin αIIb); flow direction is indicated with arrow. Scale bars: 100 μm. (D) Platelets and leucocytes adherent in the stimulated vessels after 1 h of blood perfusion. Leukocytes are labeled white with CD45, a leukocyte-specific member of the protein tyrosine phosphatase family. Red, CD31; green, CD41a; white, CD45; and blue, nuclei. Scale bar: 50 μm. (E) Platelet adhesion on vWF fibers. Arrow, flow direction. Green, vWF; red, CD41a. Adapted from Ref. (29), copyright 2012 National Academy of Sciences.

(*Figure 2C*). After 1 h of perfusion, leukocytes were also shown to attach to the lumen walls with signs of migration through the barrier into the surrounding collagen hydrogel (*Figure 2D*). Mechanistic investigation revealed the secretion of vWF by the endothelial cells on surfaces, which served to bound to platelets in the blood (*Figure 2E*), indicating that the engineered vascular model was biologically functional and responsive to externally applied signaling molecules, capable of inducing thrombosis formation.

Thrombosis-on-a-chip model generated with bioprinting

As aforementioned, while soft lithography is convenient, it has limited flexibility in fabrication of vascular patterns due to the requirement of master molds. To this end, three-dimensional (3D) bioprinting has recently emerged as a versatile technology to fabricate volumetric tissue constructs possessing complex architectures, including those that are vascularized (30-36).

Among the various bioprinting systems such as inkjet bioprinter, microextrusion bioprinter, laser-assisted bioprinter, and stereolithography (32-34), sacrificial bioprinting strategies based on extrusion have been most widely used for generation of vascularized tissue constructs at relatively high resolutions. In a typical procedure, a microfibrous pattern is first deposited in an arbitrary shape, and cast by the hydrogel matrix that is subsequently crosslinked; the initially bioprinted microfibrous network is selectively removed from the hydrogel block to induce the

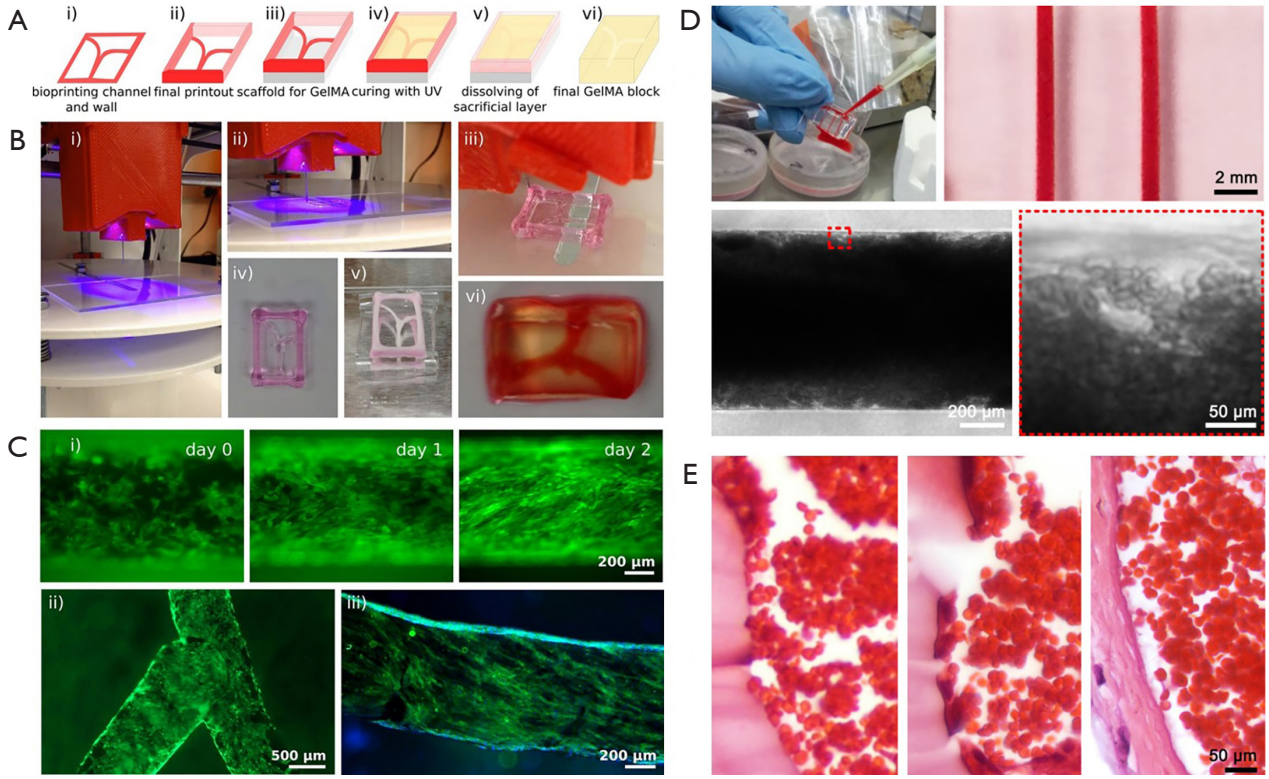

Figure 3 Thrombosis-on-a-chip model generated using bioprinting. (A) Schematic of the bioprinting process: (i,ii) bioprinting of a Pluronic template; (iii) dried template is placed on a PDMS support; (iv) the mold is filled with GelMA followed by UV crosslinking; and (v) dissolution of the sacrificial channels and frame to produce (vi) the final construct with hollow channels. (B) Photographs showing the experimental depiction of the corresponding steps of the sacrificial bioprinting process illustrated in (A). (C) Endothelialization of the hollow microchannels inside the GelMA construct for (i) a linear and (ii) bifurcating microchannels; (iii) CD31 (green) and nuclei (blue) staining of the confluent layer of HUVECs. (D) Photographs and optical micrographs showing the infusion of human whole blood into the endothelialized microchannels and the formed thrombosis-on-a-chip model. (E) Optical micrograph showing hematoxylin & eosin-stained transvers sections of (left) a thrombus without HUVECs and (middle) a thrombus with HUVECs, both at 7 days post clotting, and (right) a venous thrombus formed in vivo at 7 days. Adapted with permission from Ref. (9), copyright 2016 Royal Society of Chemistry.

formation of an interconnected microvascular network that resembles the blood vessels. The use of a bioprinting system allows significantly improved flexibility of such a method in comparison with soft lithography, as the deposition of the sacrificial template can be automated simply by altering the digital input patterns for the bioprinters. A variety of sacrificial biomaterials have been developed to enable extrusion bioprinting of vascularized constructs, ranging from carbohydrate lattices that can be removed by dissolution by perfusing medium (37), mechanical extraction of stiffened agarose microfibers (38-40), as well as thermoresponsive materials that liquefy upon temperature change, such as Pluronic F127 that transforms from the hydrogel state at room temperature to a liquid at <4 ℃ (41,42), or gelatin that gels at room temperature or lower but becomes a liquid 37 ℃ (43). Similar to soft lithography, these sacrificially bioprinted microchannels could also be functionalized with a layer of endothelial cells to introduce biological functionality.

Using a modified sacrificial bioprinting strategy based on Pluronic, we and co-workers recently reported the fabrication of a thrombosis-on-a-chip model (9). A Pluronic template, containing the vascular pattern and the outside frame, was first bioprinted and then dried in air overnight; this bioprinted structure was placed on a substrate, filled with a gelatin methacryloyl (GelMA) hydrogel pre-polymer, and photocrosslinked; eventually, the entire construct was placed in a cold buffer bath to rehydrate the Pluronic and to dissolve it out (*Figure 3A,B*). The bioprinting of not only the vascular template but also the surrounding frame

followed by desiccation served as the container to hold the hydrogel block in place, eliminating the need for additional molds. Both straight and branching microchannels could be generated using this sacrificial bioprinting method, which could be further endothelialized (*Figure 3C*). The obtained microchannels resembling the blood vessels were filled with human whole blood induced to clot, leading to formation of a biomimetic thrombosis model with aggregated blood cells (*Figure 3D*). By comparing with a human clot, the bioengineered thrombosis-on-a-chip model exhibited strong similarity when an endothelial barrier was present (*Figure 3E*). It was further demonstrated that, the acute clots in these bioprinted thrombosis models could be dissolved away by perfusing with tPA, while fibroblasts embedded within the surrounding GelMA hydrogel matrix were able to migrate into the thrombus in the absence of an intact endothelium, depositing collagens that resulted in the aging of the clot on the chip.

Conclusions

We have discussed recent advances in methods and techniques, primarily based on soft lithography and 3D bioprinting, for engineering biomimetic *in vitro* models of thrombosis. These models feature a hydrogel matrix mimicking the extracellular matrix of the biological tissue, endothelialized microchannels resembling the blood vessels, and the ability to flow human whole blood through microchannels stimulated with specific agents to induce platelet aggregation *in situ* or to directly infuse blood induced to coagulate within the microchannels. These thrombosis-on-a-chip models could faithfully reproduce this important vascular disorder *in vitro*, enabling accurate investigations into their biology and treatment. Although still preliminary, we foresee that further development of these models will eventually allow for personalized screening of intravascular interventions for treatment of thrombosis in a patient-specific manner using cells and imaging data derived from individual patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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