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***In Vitro* models for thrombogenicity testing of blood-recirculating medical devices**

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Abstract

Introduction: Blood-recirculating medical devices such as mechanical circulatory support (MCS), extracorporeal membrane oxygenators (ECMO), and hemodialyzers, are commonly used to treat or improve quality of life in patients with cardiac, pulmonary, and renal failure, respectively. As part of their regulatory approval, guidelines for thrombosis evaluation in pre-clinical development have been established. *In vitro* testing evaluates a device's potential to produce thrombosis markers in static and dynamic flow loops.

Areas covered: This review focuses on *in vitro* static and dynamic models to assess thrombosis in blood-recirculating medical devices. A summary of key devices is followed by a review of molecular markers of contact activation. Current thrombosis testing guidance documents, ISO 10993-4, ASTM F-2888, and F-2382 will be discussed, followed by analysis of their application to *in vitro* testing models.

Expert opinion: In general, researchers have favored *in vivo* models to thoroughly evaluate thrombosis, limiting *in vitro* evaluation to hemolysis. *In vitro* studies are not standardized and it is often difficult to compare studies on similar devices. As blood-recirculating devices have advanced to include wearable and implantable artificial organs, expanded guidelines standardizing *in vitro* testing are needed to identify the thrombotic potential without excessive use of *in vivo* resources during pre-clinical development.

Keywords

Blood compatibility; Blood-recirculating Medical Devices; Dynamic Thrombosis Models; Hemocompatibility Models; Static Thrombosis Models; Thrombogenicity; Thrombosis

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Declaration of interest

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1. Introduction

The development of blood-recirculating medical devices, such as extracorporeal membrane oxygenators (ECMO), mechanical circulatory support (MCS), and hemodialyzers includes an evaluation of the biocompatibility of the materials and corresponding device design. Because thromboembolism remains a potential complication from use of these devices (1), thrombogenicity testing in both *in vitro* and *in vivo* settings is an integral part of designing them for therapeutic use. Guidelines for *in vitro* thrombogenicity tests that are described in standards documents, such as ISO 10993–4 (2), vary depending on the type of blood-recirculating device being assessed. These tests provide a preliminary characterization of a device or material's thrombosis potential when exposed to blood and are usually followed with *in vivo* testing. In general, blood-recirculating devices are exposed to large blood volumes at physiological flow rates for an extended period of time. While the clinical applications for devices differ, they all require devices to remain patent over the blood exposure period. Currently, these devices can be used as bridge to transplantation or bridge to recovery therapies. As MCS, ECMO, and hemodialysis technology improves, the next generation of devices are targeted for use as wearable or implantable artificial organs to provide long term resolution to heart, lung and kidney failure, respectively. To meet this need, improved *in vitro* thrombosis testing methods are required to efficiently and accurately assess novel implantable devices.

Currently, most *in vitro* testing for blood recirculating medical devices is focused solely on determining the device's operational performance. Those that do evaluate thrombosis on the benchtop vary between studies with regards to the flow loop setup and choice of thrombosis markers. A thorough investigation into thrombosis is seldom done *in vitro*, with most studies only measuring hemolysis. Studies that use *in vivo* models commonly fail to correlate *in vivo* thrombosis testing with *in vitro* results (3,4). One of the limitations of *in vitro* systems is the requirement of use of anticoagulant, which is not always used clinically. To optimize clinical relevance, an appropriate choice for type of anticoagulant can be made and dosage can be determined based on donor specific blood coagulability to reflect the patient-specific variability in anticoagulant dosage. Some of the issues with relying on *in vivo* studies to test thrombosis is that they are costly, time consuming and require many resources. They introduce factors such as type and health of the animal, surgical techniques used, and addition of components such as grafts and connectors. Another issue with thrombosis studies in animal models is that they may not translate to humans because of differences in animal and human physiology. An ideal *in vitro* testing setup should better identify thrombotic devices early in the development process, so that researchers can avoid unnecessarily using valuable resources needed to perform *in vivo* tests.

This review will provide an overview of thrombosis testing detailed in guidance documents, such as ISO 10993–4, and the common models researchers have used to evaluate them. From a survey of thrombosis testing for recently approved medical devices, *in vitro* setups are severely underused. The case for new guidelines for *in vitro* thrombosis testing to better assess the newest generation of large, implantable and wearable blood recirculating devices is clear. Until this gap is filled, this overview of the pro-thrombotic processes catalyzed by contact with biomaterials, common molecular markers for thrombosis evaluation, and test

models that are discussed here can serve as a guide for researchers seeking to design a panel of *in vitro* thrombosis tests for a novel device. This review will first provide a summary of the important classes of blood recirculating medical devices followed by an analysis of biomaterials contact activation and the molecular markers used to determine the thrombogenicity. The review will then conclude with a discussion of the two types of *in vitro* thrombosis testing setups: static models and dynamic flow loops.

2. Device Landscape

Blood-recirculating medical devices play a large role in the delivery of modern healthcare through treating or improving the quality of life in patients with a wide range of diseases. For example, stents, catheters, vascular grafts, and heart valves are commonly used devices in cardiovascular medicine. Dialysis is commonly used to perform blood filtration in patients with impaired kidney function. The regulation of these devices by the US Food and Drug Administration (FDA) reaches back to the late 1900s. As the field has expanded, guidelines for the classification, efficiency, and safety of these devices have expanded alongside them. Advancements in the medical device field aim to improve efficiency, biocompatibility and provide longer term solutions to chronic diseases. While all blood-recirculating devices require an evaluation of biocompatibility to assess their safety, this review will focus on the thrombosis testing in MCS, ECMO, and hemodialyzers because of their relatively large blood-contacting surface areas and advancement in these technologies is increasingly moving towards the development of wearable and implantable replacement organs.

2.1 Mechanical Circulatory Support (MCS)

Mechanical Circulatory Support (MCS) is commonly used in patients with advanced heart failure. Small, continuous, flow pumps, such as ventricular assist devices (VADs) have replaced older, pulsatile pumps to improve cardiac ventricular function in the left (LVAD), right (RVAD) or both ventricles (biVAD). As VAD technology has evolved, VADs are now able to provide long-term therapies for transplant-ineligible patients (5). A review of *in vitro* testing of VADs shows that most thrombosis testing studies rely on hemolysis, leaving most of the tests called for in ISO 10993–4 to *in vivo* testing setups. This may be due to the fact that promising *in vitro* hemolysis data does not always correlate with acceptable hemolysis values *in vivo* as demonstrated by the PediaFlow 1 VAD (HeartWare International, Inc, Framingham, Mass) (6). While total artificial hearts (TAH) have been used as bridge to transplantation therapies since 1969, development on total artificial hearts was slowed by complications such as hemolysis and thrombosis (7). Recently, the temporary total artificial heart (t-TAH) and C2 Driver System (Syncardia Systems Inc, Tucson, AZ), which is designed as a bridge to transplantation therapy for patients with biventricular failure who are not candidates for LVAD placement was developed (8). In August 2018, the FDA flagged this device in a letter to health care providers for higher mortality and stroke incidents in clinical application compared to its predecessor, the t-TAH with Circulatory Support System (CSS) console.

2.2 Extracorporeal Membrane Oxygenators (ECMO)

Patients with severe cardiac or pulmonary failure are put on extracorporeal membrane oxygenator (ECMO) devices over a time period of up to several weeks during which adverse effects such as hemolysis, hemorrhage, and thrombosis are observed (9). The most common ECMO devices are hollow fiber, silicone, and poly-methyl pentene (PMP) oxygenators, which facilitate gas transfer on the membrane surface. The current standard for membrane oxygenators are the QUADROX-i® (MAQUET Cardiopulmonary, Hirrlingen, Germany) family of oxygenators. They achieve a lower pressure drop and consequently cause less hemolysis across the device than conventional cylindrical oxygenators through the arrangement of stacked polyurethane hollow fiber mats (10). Many ECMO devices are coated with a heparin coating such as the albumin-heparin BIOLINE coating available on QUADROX-i® oxygenators to reduce thrombosis formation within the oxygenator. Advances have been made in ambulatory ECMO devices such as the Paracorporeal Ambulatory Assist Lung (PAAL) as a bridge to transplantation therapy (11). Ambulatory or potentially implantable devices increase active rehabilitation and can improve patient quality of life.

2.3 Hemodialyzers

An important class of blood-recirculating medical devices are hemodialyzers, used in most patients with chronic or acute kidney failure. Chronic kidney failure (CKD) patients typically require in-center hemodialysis treatments in 3–4 hour sessions several times per week while those with acute kidney failure receive hemodialysis treatment in a hospital setting. Most hemodialyzers are composed of hollow fibers, made from polysulfone (PS), polyethersulfone (PES), or cellulose acetate and function to clear substances such as urea, creatinine, and β_2 -macroglobulin from the blood. Portable hemodialysis devices can improve patient quality of life in CKD patients. A wearable hemodialysis device, the wearable artificial kidney (WAK), is currently in the clinical trials phase of development (12). Moreover, an implantable bioartificial kidney that uses microelectromechanical systems (MEMS) based silicon membranes instead of conventional hollow fibers is under development (13).

3. Thrombosis in Response to Biomaterial Contact

In the coagulation cascade, there are two main pathways to activation, the extrinsic and intrinsic pathways. The extrinsic pathway is activated by released tissue factor upon tissue injury, and thus, does not play a large role in device-mediated thrombosis. The intrinsic pathway is composed of clotting factors present in the blood, which are activated via a series of zymogen-protease conversions to create a fibrin plug (14). While the alternative complement pathway also plays a role in thrombosis, clot formation is primarily catalyzed by the intrinsic pathway.

Within seconds of blood exposure, biomaterial surfaces rapidly adsorb serum proteins onto their surface. These proteins desorb and are exchanged for higher binding affinity proteins in a process known as the Vroman Effect. Figure 1 illustrates the pro-thrombotic events catalyzed by biomaterial contact and the Vroman pattern: albumin, immunoglobulin G

(IgG), fibrinogen, and high molecular weight kininogen (HMWK) (15). Platelets interacting with these bound proteins adhere to the material and upregulate membrane bound phosphatidylserine (16). The downstream pro-thrombotic processes from platelet activation is illustrated in Figure 2. When serum proenzymes such as prothrombin bind at this active site, zymogen-protease conversions produce the active form of the enzyme, thrombin (17-19). In platelet aggregates, thrombin amplifies the coagulation response. A positive feedback loop is created that increases platelet activation through platelet activation factor (PAF), proteinase-activated receptor 1 (PAR)1 and 4 on platelet membranes (20). Platelets also degranulate, releasing cytokines (21) and develop pseudopodia to strengthen adherence to the surface and other platelets (22). Thromboxane A2 diffuses across the platelet plasma membrane and acts as an activator for other platelets (23). Under flow conditions, platelets are captured through interaction with von Willebrand factor (vWF). This interaction is mediated through two receptors: GPIb-IX-V and platelet integrin ($\alpha_{IIb}\beta_3$). Active thrombin cleaves at least two sites on the fibrinogen molecule making non-covalent interactions between fibrinogen molecules (24,25) producing aggregated insoluble fibrin fibers. The fibers are crosslinked by Factor XIII (26) to form an aggregated structure that can trap platelets, red blood cells, and thrombin by binding at two distinct binding sites (20). The fibrin-mediated clot is susceptible to fibrinolysis via plasmin, an enzyme protease (24).

3.1 Surface Characteristics That Influence Blood-Biomaterial Interactions

Surface contact with biomaterials is one of the determining factors in device thrombosis. It is essential to consider certain surface characteristics of blood-contacting materials in evaluating thrombogenicity. While blood materials interaction is still not thoroughly understood, data has been reported on the effect of surface hydrophobicity, roughness, and charge on initiating pro-coagulatory processes.

Surface wettability, described as “hydrophobicity” or “hydrophilicity,” is generally measured by the contact angle a sessile drop makes when placed on the surface. A basic definition of hydrophobic (contact angle >90 degrees) and hydrophilic (contact angle <90 degrees) is generally accepted in the scientific community (27). Studies have focused on the influence of wettability on the protein deposition phase of the coagulation response because these interactions are defined by van der Waals and electrostatic forces (28). Plasma proteins generally have hydrophobic patches buried in their core and hydrophilic amino acids on their surface. It has been suggested that hydrophobic surfaces adsorb more proteins because the association of hydrophobic patches and the biomaterial surface is thermodynamically favorable and readily displaces water molecules. Hydrophilic surfaces generally adsorb less protein because they have strong polar interactions with the immediately contacting water layer, making it thermodynamically less favorable for proteins to displace these bonds (29). Upon adsorption, these proteins do not denature (30,31), so biological activity is generally preserved (32). Fibrinogen is able to bind in similar concentrations on hydrophilic and hydrophobic surfaces (33) and elicits a platelet mediated thrombosis when bound to a surface (34). In a study to assess protein adhesion on a potentially biocompatible coating, 2-methacryloyloxyethyl phosphorylcholine (MPC), the hydration layer created by the phosphorylcholine and water interaction was thought to be responsible for decreased protein adhesion (35).

Surface roughness, inherent to the material or defined by microstructures, can also alter the amount and type of protein adhesion on the biomaterial surface (36). However, because these features are at a larger scale than proteins, micrometer roughness most directly influences platelet activation and adherence (37). Schuster et al. compared the level of platelet adhesion on a titanium dioxide coating with varying surface roughness at the nanometer scale (38). At this smaller scale, higher roughness, along the lateral or vertical direction, correlates with a higher number of adhered platelets. This may be due to high surface roughness being more favorable for platelet anchoring on the surface or more surface area to adsorb circulating proteins, which then recruit more platelets. Hecker et al. also suggests that surface roughness may provide pockets in which platelets and fibrinogen can get trapped, forming a base for the thrombus (39).

Surface charge of the biomaterial has been suggested to be a defining factor in initial cellular adhesion and activation (40). The surface charge is defined as the tendency for the surface to acquire positive or negative charges and can be determined most directly from the chemical composition on the surface. While it has been shown that surface charge does indeed impact the initial protein layer that is adsorbed, it does not do so in a predictable manner (52). Metals like platinum, gold and silver have been implanted in the vascular system and were found induce a high level of thrombosis. It was postulated that these metals acquired a strong positive charge due to adsorption of positive ions from the blood (41-43). However, materials that have a strong anionic charge, such as glass, are also thrombogenic and are commonly used as positive controls in thrombogenicity testing (56, 57, 58). The charge density, rather than the nature of the charge may be responsible for thrombogenicity.

Surfaces can be modified with proteins to resist thrombosis. It has been shown albumin passivized surfaces prevent platelet activation (47,48) but can still interact with serum proteins when the underlying surface is exposed. Fibrinogen, unlike albumin, plays a large role in the coagulatory cascade. When fibrinogen monolayers are similarly created on the surface, they enhance activation of platelets and are shown to be highly thrombogenic (49). Plasminogen, the zymogen precursor to plasmin, which catalyzes fibrinolysis, has been targeted previously to create clot lysing surfaces. Modifying a polyurethane surface with lysine residues resulted in clot lysis activity of the biomaterial (50).

4. Molecular Markers for Thrombosis

One of the largest challenges in the evaluating thrombosis is identifying a testable molecular marker. Studies on thrombosis testing for materials used in blood-recirculating medical devices vary in which marker to test for and how many markers constitute an adequate assessment of devices' thrombogenicity potential. At the root of this problem is the current guideline for thrombosis testing, ISO 10993-4, which presents researchers aiming to conduct a thrombosis study with over ten molecular markers (Table 1). Testing each of these makers can be redundant and costly, leading to variability between studies. The result is a lack of readily comparable results from what are otherwise very similar thrombosis studies.

In addition to the ISO defined markers in Table 1, studies have also chosen to use less specific means of assessing coagulation: platelet counts, leukocyte counts, partial

thromboplastin time (PTT), and gravimetric analysis. Both platelet and leukocyte count are suitable for an overall measure of thrombosis because as they become involved in the clot, they are depleted from serum. Another method that tracks platelet activation is image analysis of adhered platelets on the biomaterial surface (51,52). Leukocytes are not directly involved in the coagulation cascade, but are integral in regulating key coagulation components. The thrombotic and inflammatory response to biomaterial contact are intricately connected through leukocytes. Engelmann and Massberg coined the phrase “immunothrombosis” to describe the large role that thrombosis plays in innate immune system defense (53). In addition to their role in the serine-protease conversion of thrombin, leukocytes directly interact with platelets to form platelet-leukocyte aggregates and fibrin to constitute the bulk of the thrombus (54). A reduction in free leukocyte count due to entrapment in platelet-leukocyte aggregates may be able to provide an overall measure of the innate immune involvement to the blood-recirculating device. In a similar mechanism to platelets, leukocyte subpopulations like neutrophils degranulate after activation within the platelet aggregate and release a variety of chemokines and cytokines that stimulate immune components, such as factors V and VIII in the coagulation cascade (55). In addition to releasing enzymes with pro-coagulant activity, leukocytes release other enzymes that break down anticoagulant factors such as heparin cofactor II (56).

A direct measure of thrombus generation is gravimetric analysis of the thrombus, where the thrombus is fixed with formaldehyde, dried, and weighed (57). However, this method only takes into account thrombus formed on the biomaterial surface.

5. Testing Methods

While the ISO guidelines provide information about the molecular markers for thrombogenicity, it offers little guidance in setting up a system to study thrombosis in blood-recirculating medical devices. A common method is a static test performed by incubation of a biomaterial with blood under a specified set of conditions. They are most often followed by testing in a dynamic flow loop designed to mimic *in vivo* conditions. These tests provide rudimentary information about thrombus formation in response to the device material and flow path. Designing these tests for new blood-recirculating medical devices involves setting up static and dynamic systems and choosing adequate molecular makers for each.

5.1 Guidelines for Choosing a Molecular Marker

There are two broad categories of blood recirculating devices: implant devices and external communicating devices. ISO 10993-4 provides guidelines for each category and must be considered in designing thorough thrombosis testing for a new blood-recirculating device. (Table 2) The first category, implant devices, include ventricular assist devices (VADs), total artificial hearts (TAH), stents, and tissue heart valves. The second, external communicating devices, include extracorporeal membrane oxygenator systems (ECMO), blood monitors, and hemodialysis equipment. The ISO guidelines for biomaterials testing does not specify which molecular marker should be tested for *in vitro* or *in vivo*. In a survey of recently approved blood-recirculating medical devices, the vast majority of studies only focus on hemolysis *in vitro* (Table 3).

While many of the markers in Table 2 are easy to test for on the benchtop, using all of them is redundant and inefficient. Markers that provide a better overall assessment of thrombosis such as partial thromboplastin time (PTT), platelet count, and leukocyte count can provide a general measure of a biomaterial or device's thrombogenicity. *ASTM-F2382: Standard Test Method for Assessments of Circulating Blood Contacting Medical Devices Materials on Partial Thromboplastin Time* details a protocol for evaluating blood contacting materials' ability to induce a thrombogenic response by measuring Partial Thromboplastin Time (PTT) (58). This test is similar to activated partial thromboplastin time (aPTT), a common clinical lab test, but does not use an activating compound. This is because the effect of the activating compound renders the test unable to distinguish between thrombogenic and biocompatible materials. When blood is collected for PTT, it is collected into citrated tubes that bind soluble calcium, which arrests further activation. (59-61). Platelet and leukocyte count tests are outlined in ASTM-F2888-13 Standard Method for Platelet Leukocyte Count—An In-Vitro Measure for Hemocompatibility Assessment of Cardiovascular Materials as a way to evaluate in vitro thrombosis response to biomaterials in static material-mediated tests (62). As the ASTM guidelines are written, a static thrombosis testing using 3.2% sodium citrate anticoagulant during exposure to the biomaterial is easily performed. The thrombosis reaction is stopped by addition of EDTA before analysis using a hematology analyzer. However, using Na-Citrate as an anticoagulant may not be sensitive enough to show a difference between positive controls and common biomaterials, but low dose heparin (2U/ml) can. For leukocyte count, neither 3.2% Na-Citrate or low dose heparin could distinguish between glass positive control and biomaterials (63). If a new coagulant must be used for platelet and leukocyte count, the standard must be updated to reflect the change.

The ISO guidelines provide several choices for molecular markers (Table 2), but they are not all used in the *in vitro* testing commonly conducted for medical devices. Overall thrombosis measures such as PTT, platelet count and leukocyte count are validated for use in static models, but need further evaluation for flow loops. Some markers, such as percent occlusion and flow reduction are more suitable for use in dynamic flow loop setups. Others, like platelet adhesion and activation markers can be easier to detect in smaller volume static systems. As we move forward with larger blood-recirculating medical devices, leaving most of the markers designated in Table 2 to *in vivo* testing will prove exceedingly time consuming and costly. Consequently, a thorough investigation of these markers in each type of system can provide a basis for updating biomaterials guidance documents. While ISO recommendations are updated, choosing an appropriate molecular marker for novel *in vitro* system is an essential step in designing an *in vitro* assay for a novel device or biomaterial. The most important consideration is the type of system (static or dynamic). Researchers should aim to test for a combination of markers that assess the different components of the coagulation cascade (platelets, leukocytes, complement system) as well as a general measure of coagulation such as partial thromboplastin time.

5.2 Blood Preparation

In both static incubation and dynamic flow loop models, the quality of the blood is essential. It should be fresh, as platelet and leukocyte function is diminished after 4 hours (64). Atraumatic blood collection, using a 21 gauge needle, is needed to minimize venostasis and

platelet activation (1). Additionally, ASTM F-2888 specifies that blood should be taken from human donors who have not taken aspirin, acetaminophen, naproxen, warfarin, heparin, or ibuprofen for ten days. These drugs interfere with the coagulation process, and therefore, diminish coagulation response. Aspirin and naproxen are part of the non-steroidal anti-inflammatory (NSAID) class of drugs that block cyclooxygenases, part of the inflammatory cascade. Aspirin is used as an anti-platelet agent because of its inhibition of downstream production of thromboxane A₂ within platelets, increasing fibrin clot porosity (65). Naproxen acts similarly through the inhibition of thromboxane B₂ (66). Systemic platelet function is only restored after platelets are replaced. Ibuprofen and acetaminophen decrease platelet activity reversibly. Warfarin is a Vitamin K antagonist that prevents the synthesis of factor X and prothrombin (67). Blood from donors who have consumed any of these drugs should not be used for *in vitro* thrombogenicity testing because of decreased coagulation activity. An important consideration for the blood used in an *in vitro* thrombosis study is the anticoagulant used and its concentration. The two most common anticoagulants that are used in *in vitro* flow loops are lithium heparin and sodium citrate. Clinically, heparin is the most common anticoagulant used in ECMO, VAD, or dialysis therapies while sodium citrate is infrequently used because it irreversibly binds calcium. In order to restore normal coagulation properties of citrated blood, calcium must be added into the blood before use in an *in vitro* test. The choice of which anticoagulant is most appropriate can depend on the requirements of the post-test analysis of blood or comparison to clinically relevant conditions. While heparin is more common, citrated blood can provide a distinct advantage for *in vitro* models because of the ease of handling and stability of citrated blood before use in a study. The level of anticoagulation for blood used in an *in vitro* flow loop should ideally be donor specific because of high variability in the coagulability of blood in different species and between donors.

5.3 Static Biomaterials Incubation

Static blood tests for thrombogenicity are conducted by incubating fresh blood or platelet rich plasma (PRP) with a biomaterial surface for a specified surface area to volume ratio at a given set of conditions (44,52). The blood is tested for pro-coagulant markers and/or the surface is examined to determine protein adhesion or thrombus formation. Static blood tests are often performed at 37°C with fresh human blood to mimic physiological settings most accurately. Parameters that are varied include type and concentration of anti-coagulant, incubation time, and surface area to blood volume ratio. Static models only provide rudimentary thrombosis potential evaluation because they cannot simulate *in vivo* conditions closely due to the lack of blood flow. The large blood-air interface in an *in vitro* test is important to consider due to the ability of blood-air interfaces to create protein aggregates, leading to non-specific platelet activation (68). By designing a closed incubation chamber, where blood is exposed to both top and bottom surfaces of biomaterial, thrombosis due to a large blood-air interface can be minimized. A disadvantage of static *in vitro* models is cell sedimentation. Because much of the thrombosis response is mediated by activated platelets, a reduction in platelet exposure to the biomaterial surface can underestimate this response. Static models are also performed for short incubation times due to diminished blood quality after 4 hours, and therefore, unable to provide long-term thrombosis

5.4 Incubation of Biomaterials with Agitation

Agitated incubation chambers provide some advantages over static models, but are still not adequate alone in characterizing the thrombosis potential of biomaterials. In a quasi-static *in vitro* model, blood and materials are incubated on a shaker so blood moves over the surface, but flow is chaotic and non-uniform. Introducing the non-static element of agitation better recreates fluid movement over the biomaterial surface, and thus, can provide flow-mediated thrombosis activation that is relevant to *in vivo* conditions (86). While the static models induce blood movement over the surface, a directed flow path with defined shear rates is not present. Therefore, it is impossible to characterize flow-mediated thrombosis due to flow path variables. An incubation chamber that is closed with another biomaterial eradicates the blood air interface and movement of blood over the surface can ameliorate the issue of cell sedimentation (87). These models still have the disadvantage of short incubation periods. Therefore, while these conditions more closely mimic *in vivo* conditions than static incubation, a quasi-static approach cannot isolate the thrombosis potential due to blood interaction with a biomaterial.

5.5 Dynamic Models

Dynamic circulation loops have been developed to evaluate the performance and biocompatibility of medical devices (88). While the flow loop design can vary, prevention of blood-air contact, mechanical stress, constant temperature and blood pH is important for reliable hemocompatibility testing (89). Physiological flow rate, temperature and flow type can be replicated with an *in vitro* flow loop. Unlike static tests, dynamic tests include another variable to thrombogenicity: flow path. Aspects of flow path, including shear rate, pulsatile or continuous flow, can influence the evaluation of the biomaterial or blood-recirculating device. Thus far, *in vitro* thrombogenicity testing under flow conditions attempt to mimic a set limited physiological features, such as temperature and pressure. These conditions vary between studies, so while this review categorizes similar flow loops together, it should be noted that these flow loops are quite varied, and drawing meaningful comparisons between thrombogenicity results for any two systems can be exceedingly difficult.

5.5.1 Flow Path Conditions—Using an *in vitro* dynamic model to evaluate the thrombogenicity of a biomaterial or medical device introduces another variable: flow path. The flow path is defined by the design of the medical device and flow loop components, and can include regions of high flow, stagnation and eddies (recirculation). A characterization of flow path through medical devices is most commonly done by computational fluid dynamics (CFD) (90,91,92). Simulations with CFD can provide insight into regions where flow-mediated thrombosis, due to patterns of stagnation or high shear rate can occur.

The effect of high wall shear rates on coagulation is well documented. Wall shear stress is defined as the amount of force per area that the near fluid layer exerts on the wall (92). Platelet adhesion to polyethylene tubing primed with normal plasma is increased in eightfold when shear rate was increased from 50 s^{-1} to 500 s^{-1} (93). At high shear rates, margination, or the process by which platelets accumulate at the vessel walls is enhanced (94,95). The platelet path of a platelet undergoing margination, caused by the presence of red blood cells

or due to high shear rate, is shown in Figure 3. The higher rate of platelet transport to the wall is termed enhanced diffusivity, and creates more opportunity for platelet activation (95). High shear rates also increase diffusion of solutes and particles, such as proteins, to the biomaterial surface (96). Protein adsorption promotes thrombus formation through the von Willebrand factor (vWF), which preferentially binds platelets and activates them at high shear rates. The GPIb ligand on platelets membranes binds vWF (97), leading to the formation of vWF nets. The platelet-vWF net subsequently forms the base of the thrombus, which grows rapidly as the net captures circulating platelets. Shear stress, then, can effectively lead to platelet injury without direct interaction of the platelet with the biomaterial (98). Additionally, high shear stress also increases the risk of embolizing a vWF-mediated clot.

The type of flow, continuous or pulsatile, affects the flow path, but the relevance of using either to better simulate *in vivo* conditions is still being debated (97, 98). Pulsatile flow may be advantageous because it has been shown to reduce stagnation regions (99). These can be caused by step-wall transitions and stenotic areas (101). The formation of stagnation regions is shown through computational fluid dynamics (CFD) modeling (102). An illustration of these stagnation regions is shown in Figure 4. The stagnation regions indicated by the circular arrows in Figure 4 are reduced in negative step-wall transitions (A) and eliminated in positive step wall transitions (B). It has also been suggested that the effect of wall shear stress is more relevant than type of flow (103). Pulsatile flow loop systems have been created to evaluate the thrombogenicity of implantable cardiac devices (104). While single pass systems are less common than recirculating systems, they have important implications for thrombogenicity testing (105). *In vivo*, coagulant factors are subject to the renal clearance after each exposure to the biomaterial. Some argue that single pass systems, because they do not allow factors to accumulate, are more representative of *in vivo* conditions. However, while single pass systems use small blood volumes and allow for the regulation of shear rate, they may not sensitive enough to detect cellular activation factors (108). Parallel plate systems are popular because, when used in conjunction with a microscope, they allow for the measurement of platelet function and adhesion in real time. These chambers also have easily calculated and predefined wall shear rates (107). Dynamic flow loops more accurately simulate *in vivo* conditions than static models can and therefore may be more useful in assessing the overall thrombogenicity of a blood-recirculating medical devices. However, several factors, including the type of flow and wall shear rates created by the system components must be considered in the design of these flow loops.

5.5.2. Common Flow Loops—The design of *in vitro* dynamic models to evaluate thrombogenicity of medical devices is currently non-standardized. A common system used is the roller pump model (45). Roller pump models generate continuous or pulsatile flow through a peristaltic pump (52,106). Because roller pump models generate flow through compression, they can cause hemolysis, which can lead to pro-thrombotic activity unrelated to the biomaterial or flow path. Zhang et al, when evaluating fibrinogen absorption on varying surface chemistries, created a flow loop with pulsatile flow using a peristaltic pump (107). Others have opted for continuous flow loops to evaluate device thrombosis. Bleilevens et al, demonstrated the ability of a twin mock flow loop to reveal changes in anti-

coagulatory state using two different brands of heparin (88). This flow loop was designed to be miniaturized so that components can be tested in multiple flow loops with blood from the same donor.

Others have approached engineering dynamic models for the purpose of evaluating thrombosis by making device-specific modifications to common *in vitro* dynamic systems such as the Chandler loop (68). This system, developed in 1958, is a closed-loop flow tube partially filled with air. Blood recirculates through the system as the entire loop is rotated. Because this system does not induce flow through a pump, the system has the advantage of avoiding pump hemolysis. However, because the system recirculates blood through a blood-air interface, blood trauma, including protein denaturation and platelet activation, can be induced (108). The Chandler loop model is not suitable for hemocompatibility testing because of the de-fouling and cleaning effect of air bubbles, which can remove adhered blood cells and platelets from the biomaterial surface (109). Free-floating, activated platelets can rapidly form platelet aggregates and produce a thrombus. Because this is a flow-mediated thrombus due to flow of the entire system and not specifically due to the device, qualification of thrombi formed from a Chandler loop system cannot provide valuable information about the flow-mediated thrombosis potential of the device.

Modified Chandler loop systems have been developed with (110) and without blood-air interfaces (68,111-113). Van Oeveren, created the Hemobile, a ball-valve model modification of the Chandler loop with no air enclosed within the tubing and a mechanical device that generates flow without compression to avoid hemolysis. When platelet counts for the Hemobile, was compared to a Chandler loop and a roller pump model, they were significantly decreased in the roller pump model and Chandler loop (114). The ball-valve mechanism allows creation of pulsatile flow with physiological wall shear stress (115). The schematic for the three common types of flow loops are shown in Figure 5.

Dynamic, *in vitro* flow models provide a distinct advantage for the thrombogenicity evaluation of biomaterial surfaces and medical devices. However, because flow loops have not been standardized and the literature reveals a large variety of flow loop designs, a meaningful comparison between thrombosis evaluations *in vitro* is difficult to ascertain.

6. Conclusion

Evaluating the thrombosis potential of medical devices is an essential part of their development. Currently, a combination of *in vitro* and *in vivo* thrombosis models are used to predict the clinical thrombosis outcomes of such devices. *In vitro* studies can be categorized into two subsets: static models and dynamic models. Each of these models offer distinct advantages and disadvantages for probing the thrombosis potential of surfaces and devices. ISO 10993-4 offers a selection of testable markers for thrombosis, but offers little guidance on their use in static tests and dynamic flow loops. The result is that *in vitro* testing is underutilized in the field of blood-recirculating medical devices. When *in vitro* thrombosis testing is done, it is highly unstandardized. Flow loops use varying materials, shear rates, and physical features and test for a thrombosis using a variety of molecular markers, most commonly hemolysis. The clinical relevance of each individual flow loop is not established,

leaving most thrombosis testing to *in vivo* testing. While current guidelines may suffice for small blood contacting devices, the next generation of medical devices, wearable and artificial implantable organs, are being challenged with high volumes of blood, flow rates, and prolonged exposure to blood. Blood-recirculating medical devices such as mechanical circulatory support, ECMO systems, and hemodialysis equipment require a more thorough investigation of thrombosis in a benchtop setting as they advance into wearable and implantable devices.

7. Expert Opinion

Understanding blood-biomaterial interactions is a foundational step to assessing thrombosis in blood-recirculating medical devices. Techniques used to understand blood material interactions, especially in regards to surface characteristics such as roughness and charge, are not comparable between studies, leading to disagreement in results. Ultimately, without a thorough understanding of the effects of surface characteristics on thrombosis, developing a material for use in a blood-recirculating device that is both highly efficient and thrombus free is quite challenging. But by focusing efforts on designing *in vitro* models, the field can better assess the blood-biomaterial interaction individually in conjunction with its role in the device.

Between studies for devices in the same category of blood-recirculating medical devices, the variability in molecular markers used for thrombosis is large. The production of many of these factors represents achievement of a certain stage in thrombosis and can be restricted to either the intrinsic or extrinsic pathways. Therefore, a comparison of the level of production of any single factor cannot possibly provide an overall indication of the activation of the system in response to entire blood-recirculating devices. In order to compensate, researchers have succeeded in evaluation of thrombosis potential by testing several markers at the *in vivo* testing stage (112). However, this approach is time consuming and the clinical relevance is not established. Additionally, many of these markers are produced only during the symptomatic phase in clinical studies, which leaves little room for intervention without symptoms (116). *In vivo* studies better recreate conditions of clinical application because many of the pro-thrombotic factors generated are cleared from the blood. Platelets, when activated, are cleared from the blood through neutrophil phagocytosis (117) while thromboplastin is cleared through the reticuloendothelial system (118). In this manner, one can say that *in vitro* conditions are a “worst case” scenario because these pro-thrombosis factors are not cleared by the liver. Another advantage of an *in vivo* system is the replenishment of nutrients and cellular energy source, glucose, which can prevent cells from becoming necrotic and losing function.

The question of how to evaluate thrombosis *in vitro*, from the molecular markers to test for to the setup is largely left to the discretion of those developing the device. The result is an extreme variability in how devices are evaluated for thrombosis, even if those devices are relatively similar. As advances in the ECMO, hemodialysis, and MCS fields approach targets for wearable devices or implantation, better models are needed to track our progress and compare these devices with traditional ones. Methods that better evaluate thrombosis *in vitro* can prevent devices that would fail *in vivo* from reaching *in vivo* testing or clinical phase

development. Identifying thrombosis issues in the *in vitro* stage will allow for more targeted changes to the device design or material, prevent unnecessary animal testing and increase the efficiency of medical device development. Ultimately, this standardized set of methods would work towards the goal of engineering more biocompatible devices that present unforeseen complications in clinical settings.

7.1 Five Year View

While the basic thrombogenicity testing methods, static incubation and dynamic flow loop models, have been long established the recent explosion in the development of implantable and blood-recirculating medical devices has catalyzed a re-evaluation of the use and design of these methods. Blood biocompatibility of the device material and design remains a chief concern in the use and limitations of these devices. As *in vitro* tests are validated for their clinical relevance, *in vitro* studies will include a more thorough analysis of blood-biomaterial interaction. In the next few years, advances in better characterizing blood-materials interactions are expected. Most importantly, as *in vitro* models are developed and standardized, the thrombogenicity evaluation of devices *in vitro* will become more predictive of thrombosis *in vivo*.

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Article Highlights

- Thrombosis testing markers vary across studies and no one testing marker can provide a complete picture of the level of thrombosis due to blood contact with a device.
- Static models can provide a method to study thrombosis due to blood-material interaction but are limited in use for evaluation of whole devices due to the lack of flow.
- Dynamic models, while allowing for an overall evaluation of thrombosis due to blood contact with the device material and flow path, are highly unstandardized.
- In 2019, nearly 60 years after the first significant dynamic flow loop, the Chandler loop, was developed, medical device thrombosis testing is still burdened by the lack of a standardized set of *in vitro* static and dynamic tests that allow for the thorough evaluation of thrombosis *in vitro*.
- As blood-recirculating medical devices become more advanced and require longer blood exposure, better and more consistent thrombosis testing models required. These studies will be able to compare and evaluate these new devices while minimizing the use of animal studies.

Figure 1:

Overview of the blood response to biomaterial contact and the Vroman Effect. When blood comes into contact with a biomaterial, several components of the immune and coagulation systems are activated. These include contact phase, complement, thrombocyte and leukocyte activation. These pathways all culminate in fibrin production. The Vroman Effect refers to the subsequent adsorption and desorption of blood proteins on a biomaterial surface. From left to right, albumin is replaced with immunoglobulin G (IgG) and fibrinogen within the first 10 microseconds of exposure. High molecular weight kininogen (HMWK) adsorbs to the surface following the fibrinogen, but the binding kinetics are much slower than the binding of platelets on a fibrinogen surface. The concentration of each protein in blood is displayed below its name.

Figure 2:

Simplified schematic of platelet activation. In response to contact with a foreign surface, platelets develop pseudopodia, degranulate to release pro-coagulatory cytokines, and upregulate phosphotydylserine. Prothrombin docks at phosphotydylserine, an anionic binding site, and is converted to its active form, thrombin. Thrombin cleaves fibrinogen to fibrin, which associates with other fibrin monomers to create fibrin fibers. Fibrin fibers, through von Willebrand factor, create platelet-fibrin networks under flow.

Figure 3:

Platelet Margination Process. Platelet path of a platelet undergoing margination. This simulation is done with a (hematocrit) = 0.40 and (viscosity ratio) = 5. (a) Platelet location along length of travel (b) end view showing radial path of the platelet Adapted from: Reasor DA, Mehrabadi M, Ku DN, Aidun CK. Determination of Critical Parameters in Platelet Margination. *Ann Biomed Eng.* 2013;41(2):238–49

Figure 4:

Illustration of stagnation regions in negative step transition (A) and positive step transition (B) in continuous flow paths from CFD modeling. Stagnation regions can lead to thrombus formation through increased local concentration of pro-coagulant proteins. Pulsatile flow can eliminate completely stagnation regions produced by negative step transitions, but only reduces the size of stagnation regions caused by positive step-wall transitions. Reprinted from: Corbett SC, Ajdari A, Coskun AU, et al. Effect of Pulsatile Blood Flow on Thrombosis Potential With a Step Wall Transition. *ASAIO J.* 2010 Jul-Aug;56(4):290-5

Figure 5:

Schematic representations of three common flow loops. The Chandler Loop (top left) has an air-blood interface that causes hemolysis and de-fouling of the circuit, making it unsuitable for thrombosis studies. The roller pump model (bottom) generates hemolysis through the pump mechanism and thus is also unsuitable for thrombosis studies. A modified Chandler Loop system, the Hemobile (top right) eradicates the blood-air interface and generates flow without compression. Studies have showed that the Hemobile generates less hemolysis than the roller pump and thus may be more suitable for thrombosis studies. These flow loops are modified for thrombogenicity testing purposes through the attachment of a medical device to the flow loop. Reprinted from: van Oeveren W, Tiellu IF, de Hart J. Comparison of modified Chandler, roller pump, and ball valve circulation models for in vitro testing in high blood flow conditions: application in thrombogenicity testing of different materials for vascular applications. *Int J Biomater*. 2012: 673163

Common Markers suggested by ISO 10993-4 for use in thrombosis testing and their function in the coagulation or complement cascades.

Table 1:

D-Dimer	A product of the degradation of cross linked fibrin that indicates fibrinolysis
F₁₊₂	Activation fragment that is released from prothrombin upon its conversion to thrombin
Thrombin Anti-Thrombin (TAT)	Anti-thrombin (AT0), a serine protease inhibitor, binds to thrombin to form the thrombin anti-thrombin (TAT) complex. Thrombin, when bound in this form loses enzymatic activity. TAT limits coagulation via inhibition of Factors Xa, IXa, XIa and XIIa
Platelet Factor 4 (PF-4)	A chemokine part of the CXC family that is released from activated platelets upon degranulation. Functions as a chemoattractant and alters the effect of heparin-like molecules
β-Thromboglobulin (β-TG)	A chemokine part of the CXC family that is released from activated platelets upon degranulation. Functions as a chemoattractant for fibroblasts
Thromboxane B2	An eicosanoid that induces platelet aggregation through altering intracellular calcium concentration
C3a	The active, soluble product of C3 cleavage. Is produced through the classical, lectin, and alternative complement pathways.
C5a	The active, soluble product of C5 cleavage. A late product of all three pathways of complement that is a potent chemoattractant for neutrophils, monocytes and macrophages
Terminal Complement Complex (TCC)	Complex assembled on the surface of membranes. Consists of C5a, C6, C8 and C9. The terminal complement complex creates pores in the cell membrane which leads to cell death via osmotic flux
Activated factor B (Bb)	A serine esterase product of the alternative pathway of complement. Cleaves and activates plasminogen and contains a von Willebrand Factor domain
iC3b	A product of the classical pathway of complement. Acts as an opsonin and is deposited on the surface of antibody-antigen complexes. This promotes phagocytosis by neutrophils and macrophages
C4d	The end product of the alternative pathway of complement. A known marker of antibody mediated rejection
sC5-b9	The soluble component of the terminal complement complex (TCC) that is the end product of the of the three pathways of complement activation

Table 2:

Molecular markers designated by ISO 10993-4 for Implant Devices and External Communicating Devices. Most molecular markers for the two devices overlap and are suggested for testing in pre-clinical development.

Test	Implant Devices	External Communicating
Percent Occlusion	x	x
Scanning EM (platelet adhesion and aggregation); platelet morphology	x	x
Flow Reduction	x	x
Labeled antibodies to thrombotic components	x	
Autopsy of devices and distal organs (gross and microscopic); histopathology	x	
Specific Coagulation Factor Assay; FPA, D-Dimer, F ₁₊₂ , TAT	x	x
PTT (non-activated)	x	x
PF-4, β -TG, thromboxane B2	x	x
Platelet Activation Markers, micro particles, count, adhesion, function analysis and aggregation	x	x
Gamma imaging of radiolabeled platelets	x	x
Leukocyte Count	x	x
Hemolysis	x	x
Gravimetric Analysis		x
Light Microscopy		x
Pressure Drop across device		x
PT,TT, plasma fibrinogen, FDP	x	x
Template Bleeding Time		x

Table 3: Selection of *In Vitro* Thrombosis Testing for Blood-Recirculating Medical Devices. Both implant devices (VADs) and external communicating devices (ECMO, CPB) were reviewed for thrombosis testing in accordance with ISO 10993-4. Most *in vitro* studies focus on hemolysis, leaving the remaining testing for *in vivo* models.

Table 3:

Type of Device	Reference	Name of Device	Hemolysis	Platelet Activation	Plasma Fibrinogen	Platelet Count	Leukocyte Count	β-Thrombo-globulin	SEM Adherence	Platelet Factor-4	PTT	Platelet Adhesion
VAD	Johnson (6)	PediaFlow 1	x	x	x							
VAD	Maul(70)	PediaFlow 2	x									
VAD	Antaki (71)	PediaFlow 3	x									
VAD	Olta (72)	PediaFlow 4	x									
VAD	Bourque (73)	HeartMate II	x									
VAD		HeartMate III	x									
Blood Pump	Muramatsu (74), Kawahito (75),Takami (76)	Gyro C IE3	x					x	x	x		
Membrane Oxygenator	Cornelius (71)	iD Pediatric Membrane Oxygenator	x	x								
Membrane Oxygenator	Madhani (11), Orizondo (72)	Paracorporeal Ambulatory Assist Lung (PAAL)	x									
Membrane Oxygenator	Kawahito (75),	Silicone Coated Polypropylene Hollow Fiber				x	x	x				
Membrane Oxygenator	Venema (80), Meyer (81)	Quadrox-i ® BE HMO 50,000	x			x						
Hemodialyzer	Roy (82)	Polysulfone Membranes	x									x
Hemodialyzer	Kung (83)	Heparin immobilized on Cellulose Acetate Membrane	x			x	x					
Hemodialyzer	Fissell (84), Ahmadi (85)	Silicon Nanopore Membranes	x	x							x	