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Rana Gbyli
University of New Haven

Anna Mercaldi
University of New Haven

Harihara Sundaram
University of New Haven

Kagya Amoako
University of New Haven, kamoako@newhaven.edu

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Achieving Totally Local Anticoagulation on Blood Contacting Devices

*Rana Gbyli, Anna Mercaldi, Harihara Sundaram, and Kagya Amoako**

Rana Gbyli, Anna Mercaldi, Dr. Harihara Sundaram, and Dr. Kagya A. Amoako

Department of Biology and Environmental Science, Chemical Engineering, and Mechanical and Biomedical Engineering, University of New Haven, West Haven, CT 06516, USA.
Department of Chemical Engineering, University of Washington, Seattle, WA 98195, USA.

E-mail: kamoako@newhaven.edu
Dr. Kagya A. Amoako

Dept. of Mech., Ind., and Biomedical Engineering
300 Boston Post Road
West Haven, CT 06516
USA

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Abstract

The recent years have witnessed an increased activity in biocompatibility research aimed at limiting biomaterial-induced blood coagulation. From 2008 to 2016, a total of $36,946,764.00 USD was awarded in grants to 213 research proposals and as large as 50.4% ($18,627,854.00) of that award monies were distributed to 101 proposals over the fiscal years of FY14 to FY16 alone. However, the complexity in blood responses to biomaterials, variability in blood function between individuals and animal species, and differences in medical device application and test setting all continue to pose difficulties in making a breakthrough in this field. This review focuses on the remaining challenges in the context of biomaterial surface interaction with blood, biomaterial properties and their influence on coagulation, old and new surface anticoagulation methods, main test systems (complement and platelet function) for evaluating those methods, limitations of modification techniques, and the current state of systemic anticoagulation usage as adjunctive therapy for controlling blood coagulation on biomaterials. Finally, we propose ingredients necessary for advancing the field towards achieving totally local surface anticoagulation on blood contacting devices including standardization of *in vitro* and *in-vivo* test methods. Some highlights of recent forward-looking work and articles on local anticoagulation are also presented.

1. Introduction

Large quantities of blood-contacting medical devices with procoagulant surfaces are used annually world-wide.[1, 2] It is estimated that more than 200 million of these devices are utilized in patients in the U.S alone.[3] They range from devices with small surface areas like catheters, vascular grafts, heart valves, cannulas, glucose, lactate sensors, and stents to those with moderate surface areas like pacemakers, artificial kidneys, and left ventricular assist devices. Then there are those with relatively larger surface areas like the artificial lungs,

artificial hearts, and extracorporeal membrane oxygenation circuits. The surfaces of these devices are made up of artificial materials that are different from endothelial cell surfaces, which interface with flowing blood.[4–6] Among other features, these cells express enzymes and secrete nitric oxide that maintains blood tone.[7–10] For example, nitric oxide inhibits clot formation while thrombolytic enzymes break down formed clots. For artificial materials, these properties are absent and rapidly activate blood into clots upon contact.[11,12] For blood-contacting devices, clot formation can cause cessation of blood flow and lead to device failure.[13–15] Moreover, devices that do not fail may release clots into the systemic circulation and cause embolic complications.[14,16–18] In life support devices these clots can result in morbidity and mortality. For instance, a small bore vascular graft serving as a coronary artery may occlude from clot formation and cause myocardial infarction (heart attack). With artificial lungs, clotting is especially problematic as they have relatively large surface areas (1.3-2 m^2) and a period of usage lasting from several weeks to months. Other clot-related problems with artificial lungs include increased device resistance to blood flow and reduced mass transport across their gas exchange membranes and they will typically fail after 7-14 days with accompanying hemorrhagic complications.[13–15,18] As a result, their usage is limited with no solution in place for permanent lung replacement. Catheters without soluble heparin locking, on the other hand, have limited a lifespan and do not reliably allow repeated sampling of blood or continuous pressure monitoring in patients as their small lumen diameters make them more prone to failure by clots.[19–22]

This review will look at some of the major anticoagulation approaches in clinical and experimental use over the last two decades. It will also focus on some of the remaining important and practical aspects of blood/biomaterial compatibility and problems with compatibility testing as well as keys to improving the blood-contacting artificial surface so that systemic anticoagulant drugs do not play a major role. Totally local anticoagulation on blood contacting devices is achievable and may be realized in the near future with robust

multifunctional artificial surfaces. These next generation surfaces will be rendered non-fouling/anti-clotting and remain so either through a single modification method that is stable to hemodynamic and hemochemical interactions or, more realistically, through multi-mechanistic techniques. The latter approach will interrupt clot formation at both the contact and propagation phases of the coagulation cascade.

2. Biomaterial Surface and Blood Interaction

Under normal physiologic conditions, a delicate balance between thrombus formation and destruction is sustained by a complex series of mechanisms and interactions among platelets, the vascular endothelium, the coagulation cascade, and the fibrinolytic system.[23] Blood remains, under healthy hemostasis, in contact only with the normally anti-thrombogenic endothelium that is lined with cells which prevent adhesion, aggregation, and activation of platelets, preclude the activation of the coagulation cascade, and modulate hemostasis through the expression and secretion of a spectrum of molecules.[24] Among these factors are prostaglandin, nitric oxide, thrombomodulin, heparan sulfate, tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA) and plasminogen activator inhibitors.[11] Thrombomodulin combines with thrombin and the resulting complex activates protein C which acts, upon its binding with protein S, as a brake on the coagulation cascade by inactivating FVa and FVIIIa. Also, heparan sulfate inhibits blood coagulation by binding and activating anti-thrombin, which is the main inhibitor of thrombin and FXa. Moreover, the tissue factor pathway inhibitor (TFPI) inhibits the TF–FVIIa complex by forming a quaternary complex TFPI–TF–FVIIa–FXa.[25] While the aforementioned factors inhibit blood clotting, t-PA, u-PA, and plasminogen activator inhibitors modulate fibrinolysis.[26] Endothelial cells also have a low surface energy that keeps blood proteins from changing conformation.[25] However, when the continuity of the healthy endothelium is disrupted, for instance upon an endothelial injury, this non-thrombogenic surface is transformed into a

thrombogenic one[24], and a thrombotic reaction starts on the cells' subendothelium matrix and on the connective tissues which get exposed[27]. Circulating platelets then adhere to these structures and start the hemostatic process.[27]

In contrast to the healthy endothelium, artificial surfaces lack such endothelial properties, and thus implants, whether intended for short term use (stents, catheters, heart valves) or long term applications (extracorporeal circulation), face a challenge, when in contact with blood, to oppose the natural coagulation process that becomes activated. Thrombus formation is a common cause of failure of blood contacting medical devices since artificial biomaterials promote clotting through a complex series of interconnected processes that include protein adsorption, adhesion of platelets, leukocytes, and red blood cells, thrombin generation, and complement activation.[24]

Coagulation on biomaterials is initiated by the contact system through the activity of three main zymogens produced by the liver. These zymogens, namely Factor XII (hageman factor), Factor XI, and high molecular weight kininogen (HK) all exist in plasma, and upon contact with artificial surfaces, they go through the adsorption, initiation, and activation phases.[11] In the activation phase, zymogen Factor XII first adsorbs to negatively charged surfaces and activates to FXIIa by autoactivation. This process cleaves a single disulfide bridge of FXII allowing its heavy chain to bind to the surface while revealing its active site. At this point, FXIIa becomes a co-factor for prekallikrein (PK) and FXI which exist in plasma as non-covalent complexes with HK (PK-HK and XI-HK). The PK-HK complex binds to FXIIa to activate prekallikrein into kallikrein. The FXI-HK complex also binds to FXIIa to activate factor FXI into FXIa to complete the activation phase of the contact system. Zymogens XII, XI, and PK at this point have been activated into XIIa, XIa and K enzymes.[11] Once activated, the contact system uses a positive feedback mechanism potentiated by kallikrein. Kallikrein acts on FXII and HK substrates to speed up their conversion into FXIIa and HKa. For example, HKa binds 10 fold faster than HK to PK and FXI thereby accelerating

the association PK and FXI. This feedback mechanism serves as the initial driver of all amplification reactions in blood coagulation starting from the contact system down to a formed clot. In addition to the positive feedback mechanism, the contact system also has a negative feedback arm to modulate itself. During negative feedback control, Kallikrein cleaves off a fragment of adsorbed FXIIa, (FXIIf), into plasma leaving behind inactivated FXIIi. Although FXIIf is still active, its participation in blood coagulation is decreased as it loses its affinity for surfaces. It, therefore, exhibits low activity for prekallikrein to kallikrein conversion. FXIa is also a player for the suppression of contact activation. It cleaves off the light chain of adsorbed HK, HKlc, which contains the active site of HK leaving behind the heavy chain portion (HKhc). This reaction terminates the cofactor activity of HK and thus allows FXIa to desorb from the artificial surface.[11] The effect of the negative feedback mechanism has, however, not inhibited the contact system adequately to prevent downstream activation of pro-coagulant factors in the intrinsic and extrinsic pathways of the coagulation system that lead to clot formation in blood-contacting devices.

Post contact-system blood coagulation has been traditionally viewed in two separate pathways; the intrinsic and extrinsic pathways. Such views have essentially faded as it is now more accepted that the pathways interact in vivo. The contact system is viewed as part of the intrinsic pathway because it initiates that branch of blood coagulation. The intrinsic pathway is associated with artificial surfaces and consists of reactions involving only vascular elements that can occur independently of factor VIIa whereas the extrinsic branch consists of blood in addition to vascular elements. The main difference between the branches lies in the way they activate FIX. The intrinsic system uses FXIa in the presence of Ca^{2+} whereas the extrinsic system requires tissue factor (TF) usually embedded in the cell membrane lipid bilayer in addition to calcium.

The intrinsic pathway is initiated by the activation of IX to IXa by Factor XIa which is activated in the contact system. FIX can also be activated by TF in the presence of a

phospholipid (PL) surface and cofactor VIIa. For the next reaction to occur, calcium, FIXa together with the tenase complex (phospholipid surface-cofactor VIIIa) must work together to activate FX. This marks the end of the intrinsic pathway.[11,26]

Activation of the extrinsic pathway is achieved again by TF in the presence of PL and VIIa, the same coagulant elements association utilized in the intrinsic pathway. As TF is present in the endothelial tissue, activation of the intrinsic pathway is usually associated with tissue damage. However, biomaterial-induced platelet activation and inflammation can also activate the intrinsic branch, as TF is also present in platelets and leukocytes. When TF associates with a PL and cofactor VIIa, FX is activated into FXa marking the end of the extrinsic pathway and the beginning of the common pathway of blood coagulation.[11,26]

The common pathway is activated by the prothrombinase complex consisting of FXa, cofactor Va on platelets' membranes and a PL surface in the presence of calcium. With FVa serving as the platelet membrane receptor for FXa, the activated platelet produces and secrets prothrombin. The complexing of these four elements to form prothrombinase increases prothrombin activity by 300,000 fold compared to that achieved by FXa and prothrombin alone. Adsorbed and activated platelets on the artificial surface, at this point, are connected by fibrinogen-glycoprotein IIb/IIIa platelet membrane receptor bridges following cleavage of fibrinopeptide A and B from fibrinogen. Platelet aggregation achieved by interconnecting-platelet- secreted fibrinogen bridges is still a premature clot until thrombin crosslinks the platelet aggregate by converting fibrinogen into longer fibrin strands that bind to activated platelets multiple times. Finally, a solid clot is formed when thrombin activates FXIII to FXIIIa to cross link the interconnecting fibrin. This creates an insoluble fibrin network which is the end point of both coagulation pathways. Thrombin has a dual role in haemostasis, as in addition to facilitating the fibrin network formation. It also functions as an activator of two distinct platelet receptors, namely the protease activated receptors (PARs) 1 and 4. It also

potentiates the clotting process by activating FV, FVIII and FXI. It is therefore viewed as the most potent clotting factor in blood coagulation.[28]

2.1. Platelet Activation

Circulating platelets are activated either after damage to healthy endothelium or after blood exposure to an artificial surface. After damage to the endothelium for whatever reason, the endothelium loses its integrity and has to remedy its environment in order to restore its vascular tone. To achieve normal haemostasis, ruptured endothelial cells secret a" sticky" protein called von Willebrand factor (vWF) to bind to collagen in the exposed sub-endothelial layer so that circulating platelets can bind to the opposite ends of vWF to form a seal at the damaged area.[11,26] Glycoprotein Ib/IX receptor on platelets membrane allows binding of vWF to activate platelets. On artificial surfaces, the contact system activation leads to the generation of FXa. This, in turn, binds to FVa on platelet membrane to activate platelets. After the initial phase where few platelets are activated, a second and massive platelet activation phase ensues. The release of clotting factors from platelets' granules during the first wave of activations acts to systemically activate substantially large amounts of circulating platelets to potentiate coagulation. For instance, Activated platelets will secrete abundant Adenosine diphosphate (ADP) and Adenosine triphosphate (ATP) from their dense granule that in turn can activate neighboring platelets via ADP and ATP sensitive receptors. ADP is a ligand to the receptors P2Y1 and P2Y12, located on the platelet surface.[29-30] Among the granule content released, those of greatest physiological importance and their platelet membrane receptors, for example, are listed in **Table 1**.

Shear rate from vascular blood flow can also influence blood coagulation. At high shears platelets undergo conformational changes and release their pro-coagulant factors including vWF, which propagate clot formation- a mechanism different from activation by chemical induction. Typical shear rates in human body range on the order of 10 s^{-1} to 1500 s^{-1}

corresponding to 1 dyn/cm^2 to 55 dyn/cm^2. The larger vessels (veins and arteries) tend to have lower shear rates than the smaller ones.[31-34] For instance, the vena cava (larger diameter) will have smaller shear rate (10 s^{-1}) than venules (375 s^{-1}) and the ascending aorta (larger diameter) will have a smaller shear rate (250 s^{-1}) than arterioles (~1500 s^{-1}). Moreover, arterial flows are of higher shear rates (125 s^{-1} to 1500 s^{-1}) than venous flows (10 s^{-1} to 375 s^{-1}). These flow variations present a range of shear rates to which surfaces of blood-contacting devices can be exposed depending on where they are implanted. In a high shear rate environment, proteins and cells are highly mobile from momentum transfer and their deposition can be swept away before they amass into macro-aggregates whereas in low shear regimes seen in some blood-contacting deivces, continuous deposition and increased surface/biofoulant interaction can lead to larger blood clots and faster coagulation effects. Case in point, it has been reported that densely networked fibrin morphology is formed on surfaces exposed to low shear rates 10 s^{-1} compared to low to no discernable fiber deposition from 25 s^{-1} to 100 s^{-1} shear rates.[35] However, with respect to platelet activation, recent experiments have elucidated new platelet aggregation kinetics that led to artirial occlusion from large thrombi formation due to local release of vWF from platelets and conformational changes of platelets.[36] These clots were formed under high shear stress arterial blood flow, and their histological analyses showed high platelet to red blood cell ratio compared to low shear venous blood flow experiments. Clearly, continued efforts to elucidate hemostatis as a function of stress regimes, composition of blood, cellular and molecular environment of the vasculature, and other factors are important. Implications of increasing our understanding here will broadly impact medicine, as acute thrombotic events can be better controlled, blood-contacting device design and testing will be better informed, and their clinical performance can be enhanced.

2.2 Complement Activation

Several polymer surfaces were reported to have a strong influence on contact activation and in consequence also on the activation of coagulation and complement systems. Much research has shown that upon exposure to blood, the artificial surface becomes rapidly covered by a protein layer from different cascades and the deposition of this layer triggers the activation of the contact, coagulation and complement pathways. For instance, the adsorbed fXII and fibrinogen constituents of this layer trigger the activation of the complement, and one of its components, C3 is prone to undergo conformational changes upon binding to that layer leading eventually to the activation of the complement alternative pathway. It has also been shown that activation products of the contact activation system (Factor XIIa [FXIIa] and kallikrein), as well as thrombin and plasmin, are able to cleave purified complement component or fragments thereof in vitro.[37-41] Recently, these early observations have been confirmed and extended, and more factors have been added to the list of proteases that are potentially able to bypass convertases and directly generate complement protein anaphylatoxins C3a and C5a respectively, leading eventually to the activation of the complement alternative pathway. Whereas the incubation of C3 or C5 with FIXa, FXa, FXIa thrombin, and plasmin resulted in the release of C3a and C5a, respectively, the coagulation factor XIIa is able to activate the complement complex C1 initiating the classical pathway. Thus, there is a crosstalk between the components of the coagulation and complement cascades, and the activation of the complement on top of the adsorbed protein layer leads to the generation of the c3a and c5a that recruit and activate polymorphonuclear leukocytes and monocytes.[38-41]

3. Biomaterial surface properties and their influence on coagulation

Immediately following the contact of blood with synthetic materials, spontaneous adsorption of proteins, a phenomenon termed “fouling” occurs leading to an advanced adverse biological response afterwards. [42] In general, antifouling surfaces are based on minimizing the

intermolecular forces of interactions between extracellular biomolecules and the synthetic surface so that cells do not adhere or an adhered cell is easily released under low shear stresses. A truly antifouling surface, also referred to as a 'stealth surface', remains undetected by cells in a biological milieu, and is therefore not prone to biofouling.[43] The interfacial energy between a surface and water is expected to play an important role in conferring antifouling characteristics to the surface. To better understand fouling phenomena theoretically, Baier related fouling with surface energy.[44] A surface is referred to as a theta surface if when glycol-proteinaceous macromolecules encounter such surface, they have the least probability of getting denatured.[44] Theoretically, minimal bio-adhesion occurs at a critical surface tension in the range[45] of 20-30 mN/m preferably between 22 and 24 mN/m which is approximately equal to the dispersive component for water.[46] In a pioneering study on the structure-property relationship of surfaces in resisting protein adsorption by Whiteside's,[47] it was reported that to resist adsorption of proteins a surface needs to exhibit four molecular-level characteristics: hydrophilicity, hydrogen-bond acceptors, do not include hydrogen-bond donors, charge neutral overall. There are very few exceptions such as mannitol groups, which resist protein adsorption although they are hydrogen-bond donors.[48] Hydrophilic surfaces such as poly(2-hydroxyethyl methacrylate) (PEGMA) have interfacial energy below 5 mJ/m^2.[49] Although the mechanism of action for PEG's nonfouling is not fully understood[50], it is generally accepted that PEG's effectiveness is due to the steric repulsion effect with loss in entropy exhibited by a PEG-coated surface when proteins attempt to adsorb.[51,52] In contrast, non-polar hydrophobic surfaces exhibit high interfacial energy with water. When such surfaces are in contact with biological media, amphiphilic biomolecules such as proteins show significant adsorption to minimize the interfacial energy of such surfaces.[49] For example, when zoospores of Ulva are exposed to alternatively patterned hydrophilic and hydrophobic arrangement of self-assembled monolayers (SAM), zoospores preferred to settle on hydrophobic patterns.[53] For effective nonfouling, in terms of

surface complexity[49] a surface can be homogeneously hydrophilic (nonfouling), hydrophobic (foul and release if elastic) or amphiphilic (to 'confuse' bioorganisms).[54,55] In addition, surface texturing can also provide useful nonfouling properties as in the case of biomimicking shark-skin inspired PDMS coating using the Sharklet AF™ microtopography.[56] Surface modification is arguably the most convenient and effective method of imparting nonfouling properties to the material. As per the criteria established by Whitesides[47,57,58] PEG is the prominent candidate for nonfouling applications.[47,58,59] However, PEG polymers are prone to oxidation and may have issues with long term applications especially in water.[60] Among several possible polymers, zwitterionic polymers recently emerged as prominent candidates.[61] Due to their strong binding with water, zwitterionic polymers are very effective nonfouling materials and have been well studied by the Lin, Ishihara, and Jiang groups.[61-64] These hydrophilic polymers can be attached to a surface to impart nonfouling behavior, and they can be attached to surfaces by various methods;[65] "graft from" in which an initiator is attached to a surface from which the polymer is grown, "graft to" in which a functional chain-end polymer can be grafted, and adsorption of block copolymers. A variety of methods need to be attempted for a given polymer as the nonfouling behavior of the modified surface depends on the graft density and thickness of the given polymer.[43] Atom Transfer Radical Polymerization (ATRP) is one the most widely used method for both "graft from"[66,67] and "graft to" techniques for zwitterionic polymers[68,69] and PEG modification.[70-72] Zwitterionic grafts typically will form brush or mushroom configurations on the polymer substrate depending on graft chain separation distance. In a brush configuration, the zwitterionic polymer grafts are in close promixity that they extend away from the substrate to repel macromolecules including proteins. In a mushroom configuration, the surface polymers chains are separated by larger distances such that they expand into non-extended dimensions. The larger average separation distance between chains provide room for macromolecules to interact directly with the surface of the substrate. Hence brushes tend to be anti-fouling while

mushrooms tend to be fouling [43]. Protein resistance of these surface give an indication about their nonfouling behavior for real world biofouling applications. It is necessary that long term studies of different polymers in blood contacting applications be performed to select materials for demanding applications.[73]

4. Surface Anticoagulation Approaches

Modification approaches developed to combat clot formation on blood-contacting artificial surfaces have focused on usage of single-mechanistic anti-clotting processes through a variety of hydrophilic coatings to prevent non-specific protein adsorption by biomaterial surface hydration, nitric oxide release from polymers to inhibit platelet activation, and most recently via dual-mechanistic experimental means. **Table 2** presents these methods, their mechanisms of action, limitations, and relevant commentary about their usage.

4.1 Limitations of Approaches

Several limitations associated with anti-clotting surface modification approaches discussed in **Table 2** still challenge the field and these problems need to be solved in order to design truly hemocompatible surfaces. And such limitations must be addressed using all plausible alternatives including methodical incremental approaches. In the following section, limitations of commonly used coatings are further reviewed.

In heparin coatings, the challenge is that each heparin polymer must first attach to an antithrombin III (ATIII) serine protease inhibitor to work. The formed heparin-ATIII complex must then binds to procoagulant thrombin to deactivate it from propagating coagulation. Realistically, this scenario does not always occur due to the surface-immobilized state of heparin. Another limitation of the heparin arises from its degradation due to stresses imposed by its biological surrounding, and it gradually loses its availability to complex with ATIII.[37,74,75]

Hydrophilic coatings ranging from Sorin to Trillium increase the hydrophilicity of surfaces and also lower surface energy. Although these mechanisms lead to minimal protein adsorption, the coatings are prone to leaching due to environmental factors which lead to loss of water retention. Similarly, hydrogel and ionic liquid based coatings are shown to be highly resistant to protein adsorption and even blood progressively lose their bioactivity. Despite notable advantages of these coatings, their acute and chronic inflammatory responses in vivo have been inconsistent. Fluoro-containing coatings, on the other hand, minimize surface energy and lower surface tension[76], and have been shown to inhibit fouling from whole blood in *in vitro* and acute *in vivo* studies.[77] *In vitro* testing result also showed that liquid perfluorocarbons can be displaced from the surface under hydrodynamic shear stress.[77]
It should be noted that while the theory of surface energies was found to be important in single protein/model surface interaction study, it is a big leap to apply only this theory to the explanation of whole blood interaction with polymers in medicine**.** Poly (ethylene oxide) (PEO), otherwise known as polyethylene glycol (PEG) is a hydrophilic polyether used as coatings on blood contacting medical devices. It is made up of a structured repeat unit (CH_2-CH_2-O) that drive surface hydration lowers protein adsorption on devices. Positive results have been shown in *in vitro* testing with some reports arguing for inconsistent results.[75] Nevertheless, polyethyleneglycol has been applied as coating for biocompatibility studies on many surfaces including polymers (polyurethanes), metals (stainless steel) and as conjugants of drugs to improve their bioavailability. It is known to increase the hydrophilicity of surfaces which in turn reduces nonspecific attachment of proteins[78,79]. The hydrophilicity is typically screened by contact angle measurements which are often correlated to resistance to biofouling. Lower contact angles (72.1° from 99.6°) have been observed from PEG-grafted thermoplastic polyurethanes[80-84] which reduced platelet and bacterial attachment and from PEG-grafted stainless steel which reduced the adhesion of bovine serum albumin and *Listeria ivanovii*[85]. Moreover, PEG has been shown to prolong the *in vivo* circulation of drugs complexed to PEG

[86]. The year 1990 marked the first of eleven FDA approved PEG conjugated drugs with the last drug receiving such clearance in 2014.

An anti-inflammatory material can be applied as non-fouling surface while others are delivered as anti-inflammatory agents. The latter technique allows proteins to passively displace preadsorbed anti-inflammatory proteins.[87] Both approaches are influenced by coating stability and more *in vivo* research is needed.

Biologically active surfaces, such as nitric oxide releasing polymers, are a relatively new approach for improving blood/biomaterial compatibility. Nitric oxide is an antibacterial and antiplatelet agent that is endogenously released into flowing blood at low flux levels (0.5 - 4 $X10^{-10}$ $mol/cm^2/min$)[88] by healthy endothelium and can be incorporated into polymers, like polydimethylsiloxane (PDMS) via chemical conjugation reactions. The goal of this anti-clotting material modification method is to attain flux levels comparable to what is produced by the endothelium and at nontoxic levels. The ability to control the delivery of nitric oxide and its duration of release continues to be under investigation, as long term release has also not been achieved yet.

A much different antifouling surface modification approach which involves endothelial cell (EC) attachment to artificial surfaces is achieved by first adding endothelial cell receptors to the preferred non-biodegradable substrate followed by EC attachment. Adequate and appropriate distribution of receptors as well as surface morphology of the substrate are aimed at mimicking the extracellular matrix to promote interaction between the ECs and EC cell receptors. Many of the difficulties associated with conjugating EC receptors to substrates remain unsolved so alternative approaches using stem cells to induce endothelialization are under investigation.[24] Biodegradable polymers such as poly(1,8-octane diol citrate) (POC) are also being used as substrates to promote endothelialization. The application of POC onto grafts (POC-ePTFE) has been shown to maintain EC adhesion and proliferation of porcine cells similar to that of the native tissues, and within 10 days the EC

was confluent, while only random patches were evident on ePTFE controls.[24] For such substrates, it is important to maintain an optimum balance between polymer degradation and cells growth and replication rates, as these factors including porosity influence the polymer's performance and endothelialization. The use of self-assembled monolayers (SAM) to study antifouling materials are a unique approach as they provide a controlled protocol for evaluating and screening for resistance non-specific protein fouling. The typical substrate for SAMs is, however, gold, which is not a medical relevant surface such polyurethane, polydimethylsiloxane, or 316 stainless steel.

Surfaces coated with albumin have demonstrated increased resistance to platelet adhesion,especially when compared to other proteins found in human blood. The protein can be covalently attached to the surfaces or selectively adsorbed onto surfaces modified with PEO-tethered warfarin which increases the surface affinity for endogenous albumin.[75] Hemocompatibility studies of albumin-coated surfaces have generally not shown success. A clinical study examining prosthesis in aortic position[89], observed similar coated prosthesis performance to uncoated controls.

On the other hand, elastin-inspired coatings which are materials containing elastin, a protein found in connective tissue has been shown to limit platelet adhesion and aggregation. However, due to its insolubility, it is difficult to isolate and purify elastin[75]. Another coating inspired by proteins is the Phosphorylcholine (PC) which mimicks phosphatidylcholine found in animal cell membranes. While surfaces with PC coating limit protein and cell adhesion *in vitro* supposedly because of their zwitterionic nature and electrically neutral state at physiologic pH, the relatively weaker van-der Waals forces that hold their monolayer or bilayer coating molecules together create instabilities[75]. PC-coated surfaces have exhibited reduced platelet adhesion *in vitro* but showed no benefit in some animal studies.[37]

Pyrolytic-carbon coating takes the concept of early carbon-coated surfaces and applies it to a modern approach. The process occurs through chemical vapor deposition in which

hydrocarbon is heated and a graphite layer is crystallized as a 'highly ordered layer of carbon atoms'.[75] Although pyrolytic-carbon coated surfaces were found to reduce platelet adhesion on vascular graft, they did not affect their overall patency rate.

Needless the say many limitations exist among these antifouling coatings, and by addressing these challenges and evaluating sound alternative approaches, while limiting the risk of bleeding associated with systemic anticoagulant usage, further improvements in blood/biomaterial compatibility can be achieved.

5. Current State of Systemic Anticoagulation Usage

Largely due to surface modification limitations discussed earlier, blood coagulation on medical devices remains a challenge yet to be solved through surface/material modification alone. Systemic anticoagulation drugs, especially heparin, are still required as adjunctive therapies with the most commonly used systemic anticoagulant being heparin although other agents such as clopidogrel, aspirin, and warfarin are also used. Heparin's role in inhibiting clots formation is multi-faceted acting through inhibiting factor Xa from activating prothrombin into thrombin. When it binds the plasma protein antithrombin III, it directly inhibits thrombin from binding to platelets, and the heparin-AT complex inactivates several coagulation enzymes including thrombin, factors IXa, XIa, and XIIa.[26, 90] Heparin slows the process of thromboplastin synthesis, decelerates the conversion of prothrombin to thrombin, and inhibits the effects of thrombin on fibrinogen, blocking its conversion to fibrin. It also causes an increase in the number of negatively charged ions in the vascular wall, which helps prevent the formation of intravascular clots. In addition, heparin can act through other serine protease inhibitors such as heparin co-factor II, protein C inhibitor and tissue factor plasminogen inhibitor. The antithrombotic action of heparin in vivo is complex, and interactions with different plasma proteins and cells play significant roles in the living vasculature.

Warfarin, a coumarin derivative, is considered among the most common vitamin K antagonists (VKA) in which it generates its anticoagulation effects by affecting six vitamin K-dependent protein factors involved in the coagulation cascade (factors II, VII, IX and X and proteins C and S).[91, 92] Warfarin and heparin work in slightly different ways, but both block the production of certain cofactors in the liver that work together to promote blood to clot. The points of action of both these anticoagulants are downstream of the onset of blood coagulation and have no effect on protein adsorption. Furthermore, these anticoagulants may compromise hemostasis and cause hemorrhage as they act systemically.[93, 94]

While Warfarin inhibits Factors II and VII of the extrinsic coagulation pathway, and Factors IX, X, and prothrombin of the intrinsic pathway, heparin exerts its major effect via antithrombin, converting antithrombin to a more efficient inhibitor of circulating thrombin (factor IIa), factor Xa, factor IXa, factor XIIa, and kallikrein. However, due to the multiple anticoagulant mechanisms of heparin, differential molecular weight-based clearance, heparin resistance, and patient-specific characteristics (age, weight, gender, and tobacco), attaining therapeutic anticoagulation is complicated. Moreover, the resultant under- or over-therapeutic anticoagulation is associated with increased risks of ischemic and bleeding complications. Therefore, it is important to maintain heparin anticoagulation within a relatively narrow therapeutic range.[95] Also Heparin-induced thrombocytopenia (HIT), a condition whichdecreases platelet count during or shortly following exposure to heparin[96], is considered the most frequent drug-induced type of thrombocytopenia and can lead to significant morbidity and mortality if unrecognized. Heparin's interaction with circulating platelets lead to their activation followed by the release of their pro-thrombotic platelet-derived micro-particles, which causes platelet sequestration and consumption, and eventually thrombocytopenia.[96,97]

In surgical procedures requiring cardiopulmonary bypass (CPB) or other blood contacting devices, heparin is used to elevate the activated clotting time(ACT) increasing the

activity of the anti-thrombin. Since some patients are AT deficient and thus have a diminished heparin response, and accordingly fail to reach therapeutic ACT, the majority of them may require AT replacement often with antithrombin III (ATryn, recombinant antithrombin III [rhAT], rEVO Biologics).[98,99] In the same way, although Warfarin inhibits the activation of the vitamin K–dependent clotting factors (Factors II, VII, IX, and X) and regulatory proteins (proteins C, S, and Z), it is one of the leading drugs implicated in emergency room visits for adverse drug reactions.The potential bleeding risk associated with its therapy have led to its limited use especially in the elderly population. Currently, there exists no optimal dose[100] as it is difficult to administer warfarin at the correct dose because of its narrow therapeutic index, its tendency to cause bleeding, and the individual variability in patient response. Therefore, achieving safe and effective doses of warfarin therapy is both an urgent and important concern.

Recent research has focused on pharmacogenomics accounting for individual variation in anticoagulant activity of vitamin K antagonists.[101] Attention has focused on single-nucleotide polymorphisms (SNPs) of genes that encode two proteins: the cytochrome P450 2C9 enzyme and vitamin K epoxide reductase complex (VKORC1).[102] Studies suggest that CYP 2C9 influences warfarin metabolism and affects warfarin half-life, whereas VKORC1 plays a role in the pharmacodynamic response in expression of the enzymatic target of warfarin. For instance, patients who carry CYP 2C9*2 and CYP 2C9*3 alleles tend to require lower warfarin maintenance doses because of their slowed metabolism compared with patients who carry the “wild-type” allele.[103,104]

Aspirin and clopidogrel are also among the commonly used systemic drugs to inhibit platelet activation. Asprin irreversibly inhibits the synthesis of platelet- dependent thromboxane A2 formation and prostaglandins inside platelets and clopidogrel binds to platelets ADP receptor and P2Y12, to block ADP from activating platelets. They inhibit the clotting cascade at the biomaterial’s surface and inside platelets in an irreversible manner and

increase the risk of bleeding.[15,93] Many other studies report significantly higher postoperative bleeding complications among patients with continued antiplatelet therapy with clopidogrel.[105]

Aspirin is the "gold standard" antiplatelet agent for the prevention of arterial thromboses. The optimum dose of aspirin as an antithrombotic drug can differ in different organ circulations. While 100 mg/day is sufficient for prevention of thrombus formation in the coronary circulation, higher doses may be required for the prevention of vascular events in the cerebral and peripheral circulation. However, any effective antiplatelet dose of aspirin is associated with an increased risk of bleeding and therefore, the individual benefit/risk ratio determines the administration of the compound.[106]

The response to these systemic drugs differs from patient to patient. In fact, many patients are resistant to the effects of a single oral anticoagulation drug, so a therapy that embodies multiple drugs having different mechanisms of action is often used. One example of this is the use of a multi-targeted antithrombotic approach, involving anticoagulation (bivalirudin and warfarin) and antiplatelet therapy (dipyridamole and aspirin), in order to mitigate the pro-coagulative effects of mechanical circulatory assist devices, particularly those that are associated with the CardioWest[107] temporary total artificial heart.[108, 109] Still, the problem with this approach is that although the mechanisms of action for current antiplatelet agents are known, their combined effect may induce anticoagulation at magnitudes too large to allow restoration to normal hemostasis. The administration of these agents concomitantly can increase to risk of bleeding and lead to other unforeseen complications.[108] Careful monitoring of this variant multisystem approach using tools such as efficacy tests, safety tests, and warfarin genomics is therefore important for maximizing the therapeutic actions and minimizing the bleeding risks that are associated with the technique. Ultimately, systemic drugs increase the risk of bleeding complications because of their prolonged half-lives. Bleeding complications become even more devastating in patients with renal insufficiency

who cannot efficiently clear these long lasting drugs. The effects of systemic anticoagulation swing from bleeding in cases where it overwhelms hemostasis to device clotting when inadequate concentrations are used. In devices with large surface area, both effects (bleeding and depletion of systemic clotting factors and platelets) can occur.

Bleeding complications from systemic heparinization are the most common and life-threatening. For example, the Michigan ECMO experience with neonatal mortality is one that can still be improved if bleeding complications due to systemic anticoagulation are reduced. According to the most recent report on the impact of ECMO on neonatal mortality[109], bleeding is the most frequent cause of death in newborns managed with extracorporeal life support. In this population, intracranial hemorrhage occurred in approximately 13% of neonates, 5% of pediatric patients, and 4% of cardiac patients. Clotting complications are also common, occurring in 26% of patients.[110]

Conventional anticoagulants have somehow proven efficacy. However, due to the narrow therapeutic window, the necessary need for regular monitoring, and other complications, over the last decade or several decades, new oral anticoagulants (NOACs) have been launched and developed.[111] These new oral anti-coagulants range from Factor Xa inhibitors, such as apixaban and rivaroxaban, which selectively and directly bind to the active site of factor Xa and thereby inhibiting both free and clot associated factor Xa, to direct thrombin inhibitors (DTIs). DTIs inhibit the intrinsic activity of the thrombin. Unlike heparin, which also inhibits thrombin, the DTIs do not require a factor and can inhibit thrombin directly. These drugs, such as bivalirudin, are used for prophylaxis and treatment of venous thromboembolism and acute coronary syndrome, and for prophylaxis of thrombus formation in non-valvular atrial fibrillation.[112,113] They are also used as anticoagulation alternatives in the setting of HIT.

These new oral anticoagulants show advantages compared with conventional anticoagulants in the prevention and treatment of patients with thromboembolic events,

especially because of lower rates of hemorrhagic outcomes [111-114], the rapid onset and offset, short half-life, predictable therapeutic effect and wider therapeutic window in addition to the need for no lab monitoring. Nonetheless, there are some conditions for which parental anticoagulants remain the drug of choice. Despite the aforementioned advantages of NOACs, these drugs are not ideal because their use is limited or contraindicated under some circumstances. For example, none of the direct NOACs are approved-to-use drugs during pregnancy or in babies and children.[111, 114] Additionally, NOACs have not yet been applied in patients with mechanical mitral valve issues (with increased rates of thromboembolic and bleeding complications),[111, 114] patients with malignant disease, and those with antiphospholipid syndrome, which is associated with a greater risk of thrombophilic states.[111,114] More studies are still required to evaluate and further assess the efficacy of NOACs.

6. Keys to Achieving Totally Local Surface Anticoagulation

To advance the current status of surface anticoagulation it is critical that: novel surface modification approaches are actively pursued, in-clinical-use and promising experimental techniques are critically reviewed, limitations of the primary mechanisms of action of those modification methods are clearly established, the potential for improving on those limitations through reasonable optimization processes is assessed, and synergy benefits from co-localization of two or more anticoagulation properties on surfaces, an approach arguably of most promise and which logically transitions from proven single mechanistic methods, are evaluated. These are essential factors that need to be addressed in order to advance surface anticoagulation research.

As discussed in preceding sections, there are several ways of organizing surfaces which interact with blood and many are actively being researched. These surfaces have been designed to remain passive and hydrophilic, active and hydrophilic, or active and anti-thrombogenic. On such designer substrates, we either rely on surface hydration to keep

procoagulant blood factors away from settling on the surface or on coupling of soluble proteins (e.g. non-dominant form of antithrombin III, beta-antithrombin III)[109] and enzymes to surface-bound active agents such as heparin to inhibit activation of key procoagulant elements like thrombin and factor X. There are also catalyzing surfaces such as copper (II) doped polymers which catalyze the dissociation of nitric oxide from endogenous organic compounds such as S-nitrosothiols[115- 117] (NO donors). In addition to these designer surfaces, tailored polymeric matrix composites that secrete anti-clotting agents such as nitric oxide to limit the activation of key procoagulant platelets have been extensively studied.[118- 123]
Due to the limited and generally short-lived benefits of surface modification approaches, as deducible from the literature, an easier argument to make is that in-clinical-use approaches are either not multi-inhibitory/adequate enough to keep blood-contacting surfaces free of clot formation driven by redundantly complex set of reactions, or not robust to prevent loss of effectiveness during weeks-long (> 2 weeks) exposure to blood flow. Nonetheless, it is difficult to confidently conclude whether it is the case of adequacy or loss of function because of the lack of proper scientific data. Some experimental methods, on the other hand,continue to show outstanding resistance to nonspecific protein adsorption from the whole plasma [124-127] and in some cases to whole blood coagulation[77] although only in *in vitro* and *in vivo* preclinical settings. For these surface modification techniques, their limitations have been limited to acute *in vivo* studies at best. [77, 128-143]

Generally speaking, the optimization approaches to ensure the long-term function of in-clinical-use and experimental approaches can be grouped into fortification processes that impart stability to designer surfaces and controlled release of anti-clotting agents from matrix composites. Optimized density grafting, covalent attachment strength between graft and substrate materials, and reliable and appropriate measurement techniques to ensure ultra-low fouling graft coverage, and stability against typical hemodynamic shear stresses presented to implantable medical devices are needed in the fortification process of designer surfaces.

Microenvironment control of matrix composites for tunable release of anti-clotting agents will also play a major role in the optimization processes needed overcome current limitations of blood-contacting surfaces.

Until then, it is reasonable to question whether single-mechanistic modification methods can be fully optimized. And after fortification and micro-control of release agents, can each method alone lead to totally local surface anticoagulation? As research leads us to making such determination in the future, the potential of drawing synergy from multiple anti-clotting agents must not be overlooked. The argument can be made for pushing the boundary of surface anticoagulation research beyond the norm of single-mechanistic anti-clotting surface modifications because of millions of years of evolution which have led to equipping the luminal surface of healthy blood vasculature with several anticoagulation functions all working in concert to maintain blood tone.

The significance of this approach is that there is already a wealth of knowledge about many single-mechanistic surface modification approaches and their synergy can lead to increased hemocompatibility of blood-contacting surfaces. Even a minor improvement in the hemocompatibility could result in large upgrades in the patient-care environment.

In the following section, the mechanisms of action of a subset of the modification approaches discussed earlier are assessed in pairs and triplets to draw otherwise not obvious conclusions about their potential synergies toward developing a super anti-clotting material. For brevity, only a few highly promising and in-clinical-use methods were included in our analyses in **Table 3**. The anticoagulant functions of nitric oxide, zwitterionic coatings, polyethylene glycol, and heparin coatings are paired and tripled and the resulting multi-mechanistic action against clot formation are presented. NO's presence on any of the combinations adds the property of inhibiting platelet adsorption and activation. For example, NO release function added to either zwitterionic[116] or PMEA[117] grafted surfaces reduce the adsorption of key clotting elements (fibrinogen and platelets) at a significantly higher level than surfaces

modified with either NO release or coating alone. Zwitterionic polycarboxybetaine coated surfaces not only resist nonspecific protein adsorption much like polyethylene glycol, but also provide immobilization sites for biomolecules.[127] Heparin's action, on the other hand, is unique as it indirectly inhibits the procoagulant function of soluble thrombin. In theory, the ideal surface should effectively inhibit thrombin generation, platelet activation, and fibrinogen adsorption. This is achievable, also in theory, by blocking all contact system proteins and thus preventing the activation of the blood coagulation cascade. This method of non-specific protein resistance is an active and well-funded approach which has resulted in reduced blood activation although surface induced clot formation is still a clinical challenge. Effective inhibition of thrombin generation, platelet activation, and fibrinogen adsorption could also be achieved by designer surfaces that target each of those procoagulant elements. Such a surface could present heparin and some hydrophilic coating and NO release to blood flow.

It is important to point out that as almost all in vitro and in vivo hemocompatibility assessments of surface modification methods are done in the presence of anticoagulants, most of the blood-compatibility results are not separated from the effects of anticoagulation. Moreover, both quantitative and qualitative metrics of the effects of these anticoagulants, which are administered at known boluses and infusion rates, must be obtained from test systems that span relevant operating parameters of blood-contacting devices such as flow, surface area, duration of blood/biomaterial interaction, and geometry of the interacting surface and material. However, due to the wide variation in surface area and geometry, materials, flow regime and duration of interaction, metrics observed from the application of many of these anticoagulants may not be truly comparable from study to study. A presentation of analyses of their influence on hemocompatibility could accurately be performed largely on a case by case basis and such exercise would yield a narrative no different from that of the general state of study to study organization of anticoagulation science, which could be argued to have contributed to the challenge of gaining deeper insights into their functions and

comparison. Thus, such analyses have not been included in this review. However, its importance is acknowledged here by discussing the influence of Citrate commonly used in *in vitro* studies, on coagulation properties. The effects of citrate, including those of other anticoagulants, are increasingly be analyzed by thromboelastography (TEG) to monitor blood behavior and patient's hemostasis more and more during surgery. Throbomboelastograhy mainly measures coagulation time parameters such as R-time (kaolin activated) or TEG-ACT (tissue factor-TF and kaolin activated), K-time, α angle, maximum amplitude (MA) of tracing, G-value etc. These parameters in the order listed track accumulation time of clotting factors (R-time and TEG-ACT), kinetic time for fibrin cross linkage to reach 20 mm clot strength (K-time) or fibrinogen and platelet numbers, platelet number and function, calculated clot strength (dyne/cm^2) of the entire coagulation cascade. Fibrinolysis could also be analyzed with TEG by measuring clot lysis at 30 minutes following MA (LY-30).
TEG analysis of citrated blood compared to fresh samples from healthy human subjects (**Table 4**) indicated that duration of storage of citrated blood had no effect on TEG analyses of non-activated or citrated blood activated with kaolin or TF. TEG analyses of fresh non citrated blood were different from citrated samples. Citrated samples yielded results that were consistent with a hypercoagulable state. Morever, using kaolin to activate citrated samples generated results similar results to those obtained from fresh samples. TF gave different results[133].

Another caution to the reader is the fact that many, but not all, experimental surface anticoagulation approaches has been traditionally evaluated using single protein and static condition techniques that do not represent the complexity of blood plasma which contains many clotting factors that interact with surfaces in a time dependent matnner. Therefore their effect on blood may or may not be immediately evident. Moreover, many of these studies are not conducted under flow conditions typical around deployed blood-contacting devices.

7. Standardization of *in vitro* and *in vivo* Test Protocols

Hemocompatibility of artificial surfaces has been traditionally evaluated by analyzing the surface (protein fouling, platelet adsorption, and clotting factor deposition), accessing the material property and function based on its intended application (e.g. gas exchange efficiency, detection specificity and accuracy), and the state of the interacting biological media (complement activation, platelet function, and white cell counts) perhaps where more work is needed. As the hemocompatibility of an artificial surface is a function of blood and the interacting surface, variations in these independent variables can influence hemocompatibility outcomes and thus must be included in experiment planning in order to obtain a complete picture of how a surface will perform in clinical trials. The flow chart in **Figure 1** is our attempt to guide the decision making for hemocompatibility testing and perhaps add to the pre-clinical standardization platform greatly desired by the field. Bulk and molecular variables of blood such as shear stress, flow regime and stagnation, blood deficiencies, fouling, platelet function, and inflammation play important roles in surfaces that interact only with blood (for example gas exchange membranes), as well in surfaces that contact both blood and tissue (vascular graft, stents, and heart valves). In the latter surface group, however, cell viability, proliferation, cytotoxicity, and fibrous capsule formation are additional cellular factors to be considered. Variables as a result of bulk and molecular level properties of the material or device should also be scrutinized, and they include surface geometry, surface area to volume ratio, porosity, wettability, material-tissue compliance matching, biodegradation rate if degradable, toxicity, irritability, sensitivity, and carcinogenicity.

Accordingly, medical devices and biomaterials must undergo rigorous testing, and testing models, whether in vitro or in vivo, that is as similar as possible to the clinical application environment. Although animal models are considered expensive and time consuming, they are needed to bridge the gap between the lab and the clinic. Animal selection for in-vivo studies

should be based on the advantages and disadvantages for human clinical application. Importantly, it has been shown that the composition of blood differs considerably between various species, which leads to over- or under estimation of human blood reactions to biomaterials.[136,137]

Overall, due to the complexity of blood- biomaterial surfaces interactions and to the variability in results interpretation among different individuals and species, the clinical relevance of many studies is still a matter of debate and pose difficulties in standardization. As the number of publications in the biomedical field increase, the concern for quality assurance of their results also increase. Since the standardization of materials and devices testing is crucial and urgent, the International Organization for Standardization (ISO) provides standards for "Biological evaluation of medical devices" (ISO 10993) although test settings still vary. It is a 20-part series for evaluating the biocompatibility of medical devices prior to clinical testing. Evaluation of the biocompatibility and bio-functionality of materials is conducted by methods based on the assessment of cytotoxicity, mutagenesis/carcinogenesis, and cell bio-function. Blood-contacting medical devices and biomaterials have to be tested according to part 4 of the ISO 10993 standard.[138]

The ISO 10993-Part 4 standard is applicable to external communicating devices, either with an indirect blood path (e.g. blood collection devices, storage systems) or in direct contact with circulating blood (e.g. catheters, extracorporeal circulation systems), and implant devices (stents, heart valves, grafts). Testing, according to this standard, should be performed for five categories, based on primary processes: thrombosis, coagulation, platelets/platelet function, haematology and complement. For each contact category, primary (Level 1) and optional (Level 2) tests are recommended. Since testing should simulate clinical conditions as much as possible, most devices should be tested with heparinized blood under circulating conditions.[137]

Regarding hemolysis testing, for devices having direct contact with circulating blood, it is recommended that both direct and indirect (extract) methods for material/ surface-mediated hemolysis be conducted per ASTM F756 “Standard Practice for Assessment of Hemolytic Properties of Materials,” or an equivalent method. For hemolysis testing of devices having indirect contact with circulating blood, it is recommended that only an indirect (extract) method be conducted per ASTM F756 or an equivalent method. However, for devices or device components that do not have direct or indirect contact with circulating blood, this testing is generally not needed. For some devices where high shear stress due to blood flow may be an issue, dynamic hemolysis assessment under clinical use conditions may also be important. While ISO 10993-4 is the Hemolysis standard, it can be assessed by any of several validated methods to assay hemoglobin in plasma to obtain a hemolytic index assessed by measuring hemoglobin at 1, 2, and 4 hours by spectrophotometric. The hemolysis over this period is expressed as a percentage of positive control.[137]

Complement activation is the most relevant immunology test for devices exposed to circulating blood. An increase in a downstream complement component over baseline levels indicates activation of the complement cascade. Acceptable complement activation limits have not been established, but comparative data are valuable. For in vitro complement activation testing, the assessment of C3a, C5a, TCC, Bb, iC3b, C4d, SC5b-9 fragments activation using an established ELISA test method is recommended. Complement activation testing whether, in vivo animal models, in vitro “static” methods such as ASTM F1984 “Standard Practice for Testing for Whole Complement Activation in Serum by Solid Materials,” or in vitro dynamic testing using simulated clinical flow conditions can be used if accompanied by appropriate validation information.

A device's effects on blood coagulation may be measured in vitro by determining the rate of clot formation or the partial thromboplastin time of plasma exposed to the biomaterial

or device during an incubation period. Flow conditions, controls that are being used, and time of exposure, and the type and concentration of anticoagulation used for in vitro testing, may depend on the test system and the clinical indication of the device.[137]

Thrombosis may be addressed by performing either an in vivo or ex vivo test. The Lee White clotting-time test is also sometimes used to satisfy the requirement for a test in this category. If only a portion of the device is being utilized for thrombogenicity testing, it must be representative of all materials and important geometrical/surface features that would have direct contact with the blood. Thrombin-Antithrombin Complex (TAT), Partial Thromboplastin Time (PTT) are also used as a pseudo substitute for in vivo thrombogenicity testing. Some of in vitro assessment of thrombogenicity (e.g.the effect of extractables and leachables on platelets and the coagulation system) may also be needed for devices with indirect contact with blood.

However, a major problem regarding the use of the ISO 10993/4 standard is the lack of acceptance criteria and reference materials. There is no totally satisfactory biological test that can evaluate standard polymers. There are enormous variations between in vivo and in vitro effects, and these vary too, in different animal species. Although the ISO and the World Health Organization has published some trials and standards, it is not universally applied although significant improvements to this standard are underway and being overseen by the ISO committee.

Overall, because of the complex nature of blood compatibility, a standardized in vitro hemocompatibility test panel is needed and should enable the detection and elimination of undesired and excessive material-induced thrombosis and inflammatory events at an early stage of biomaterial development. Also, since the approval by the currently recommended panel of tests does not guarantee clinical device/biomaterial hemocompatibility, there is a

major need for improved in vivo and predictive in vitro test setups. Unsolved concerns also include the lacking standards for anticoagulation or reference materials.

8. Highlights of Promising Anticoagulation Works

A few surface modification approaches that impart multi-mechanistic anti-coagulation property have shown highly promising findings. Platelet and fibrinogen adsorption on NO transferring polymethylethylacrylate coated PU[115], as well as NO transferring PDMS grafted with polycarboxybetaine[69] have been quantified and shown to highly limit fouling. Similar anti-clotting activity has been demonstrated with NO-heparin conjugates.[139,140] The NO-PMEA and NO-pCB work were conducted on PU and PDMS respectively using flowing NO gas. In both cases, the PU and PDMS polymers simply transferred NO from a gas supply while either PU, PVC, or silicone rubber-PU copolymer was used to study the effect NO-heparin conjugate on clotting studies. None of the heparin-NO conjugate studies have used medical grade PDMS as a substrate while PMEA-NO and pCB-NO work have only transferred NO gas through PDMS. With the pervasive use of PDMS in many blood-contacting devices due to its biocompatibility and manufacturability[77,141], there are still no studies that have examined the antifouling properties of NO release with hydrophilic coatings on this material. Such materials are easily applied via dip coating to surfaces of many blood-contacting devices including artificial lungs, stents, dialysis membrane, and heart halves. Antifouling studies using perfluorocarbon (PFC) coatings[77] have been shown to exhibit nonfouling properties when exposed to blood. PFC is an FDA-approved Teflon-like material widely used in medical applications including liquid ventilation for infants and blood substitution. A recent *in vivo* anti-thrombogenic study of PFC-coated surfaces reported that the presence of PFC prevented clot formation in pigs for eight hours under high blood flow rates[77] and without soluble heparin. Another approach geared towards fast screening for highly protein resistant coatings uses standard poly (ether sulfone) substrates onto which test

polymers are conjugated through photo-induced graft polymerization using a library of monomers.[131] Such a screening tools are needed to slingshot the discovery of thromboresistant surfaces. Devising a practical method for easy attachment of fouling resistant coatings on medically relevant materials including metals is one of the holy grails in antifouling surface research. Therefore, the use of a catecholic initiator, for example, dopamine which was inspired by mussel adhesive proteins, for surface-initiated polymerization from metallic surfaces to create antifouling polymer coatings was received with enthusiasm. This approach continues to impact surface modification research as the catecholic anchor has been used in many surface grafting methods. [142]

9. Conclusion

Interest in surface modification for improved blood-biomaterial interaction is higher now than a decade ago with more federal funding being poured into this effort. This has in part led to the highly promising antifouling or antithrombogenic surface developments although these newer interfaces alone have not yet eliminated the need for systemic anticoagulation. Thus the risk of life-threatening bleeding complications is still a challenge. These newer surfaces have focused on single-mechanistic and non-integratie anti-clotting processes using hydrophilic coatings to resist non-specific protein adsorption, nitric oxide release from polymers to inhibit platelet activation, and most recently via dual-mechanistic experimental means. Dual and multi-prong are integrative approaches that draw not only on the anti-clotting property of each mechanism but also on potential synergies among the approaches for developing a super anti-clotting material. More work is needed in this area to understand how the interactions between two or more anti-clotting mechanism would affect blood coagulation.

Clear and comprehensive standards are also needed for a consistent evaluation of these surface modification approaches or after their application to devices for easier assessments

from study to study at both pre-clinical and clinical phases of testing. Factors that will drive the nature and rigor of pre-clinical testing should focus on the types of interaction between host's biological media and surface, duration of contact in the clinical setting so an appropriate animal model is selected for testing.

So the question of whether totally local anticoagulation on blood-contacting surfaces is achievable is one that we can definitely address by evaluating all potential solutions.

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Author Photograph(s) and Biographies

Rana Gbyli is a graduate student in the cellular and molecular biology program at the University of New Haven. She has a bachelor degree in Biotechnology Engineering from University of Aleppo, Syria. She is currently working as a research assistant in the Biomaterial and Medical Device Innovation Laboratory.
Her thesis work focuses on developing and characterizing nitric oxide releasing polymers, as well as investigating Nitric oxide effects on bacterial and mammalian cells growth and proliferation.

Anna Marie Mercaldi is an undergraduate student studying chemical engineering at the University of New Haven and a research assistant at the Biomaterial and Medical Device Innovation Laboratory. She began research in 2016 with lab in which focuses on nitric oxide multi-functional materials such as biomaterial NO delivery and multi-modal surface focused anti-coagulation. She is a teacher's assistant for freshmen engineering courses and an active member of Society of Women Engineers.

Dr. Kagya Amoako is an Assistant Professor of Biomedical Engineering at the University of New Haven and director of the Biomaterials and Medical Device Innovation Laboratory. He has authored several publications in journals including Advance Material Interfaces, American Society of Artificial Internal Organs, Biomaterials, and Journal of Materials Chemistry B. His research currently focuses on Nitric Oxide-based multi-functional materials under the following areas: A) Biomaterial NO delivery, B) Controlled release of NO from biomaterials, C) Bactericidal properties of NO, and D) Multi-modal surface focused anti-coagulation.

Table 1. Agonists secreted from platelets' dense** and alpha* granules including other potent agonists and their platelet membrane receptors

Agonist	Receptor
**Adenosine diphosphate (ADP)	P2Y12 receptor
**Serotonin	5-HT2A receptor (Serotonin receptor)
*Platelet activating factor (PAF)	PAF receptor
*vonWillebrand factor (vWF)	glycoprotein Ib/IX
*Platelet factor 4 (PF4)	PF4 receptor
*Thromboxane A2 (TxA2)	TxA2 receptor
*Thrombin	Protease-activated receptor -1(human)
Collagen	glycoprotein IV
Fibrinogen	glycoprotein IIb/IIIa

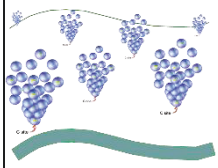
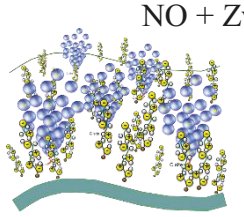
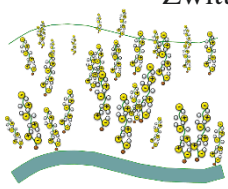
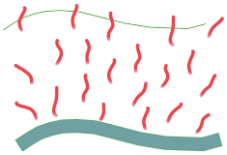
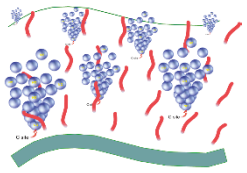
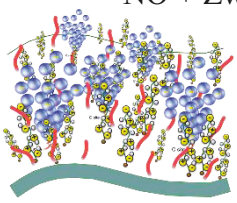
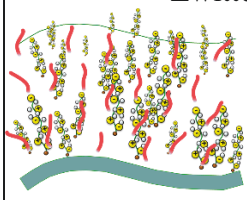
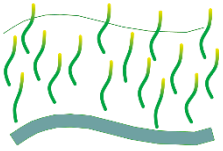
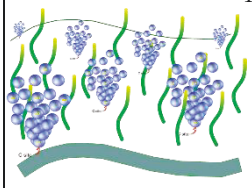
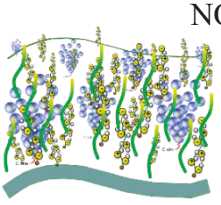
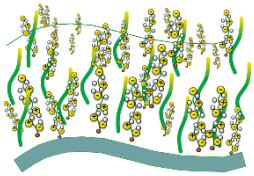
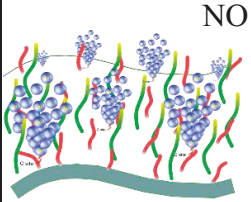
Table 2. Surface modification methods for anticoagulation on blood-contacting biomaterials.

Coating	Mechanism	Limitations	Comments
Heparin[37,74-77]	Heparin binds to ATIII and to form complex. Thrombin then attaches to complex and loses its function	Limited functionality due to reduced mobility and interaction with ATIII	Insignificant in infants or patients with ATIII deficiency
Hydrophilic coatings (E.g. Sorin, phosphorylcholine, trillium, poly(2-methoxyethlacryalt) [37,75]	Increase hydrophilicity and lower the surface energy of biomaterials; limits protein adsorption	Prone to leach and loses water retention during flow	
(Self-assembled monolayers) SAM coatings[77]	Serves as model surfaces where alkyl groups are attached to metals (usually gold) to allow attachment of polymer of interest.	Difficulty in forming ordered monolayer. largely limited to model to gold model surface	Important screening tool for new polymers
Biologically active coatings (E.g. nitric oxide)[37,75]	Antibacterial and antiplatelet agent	Toxicity; instability (storage and delivery complications)	Long term biomaterial self-release has not been achieved; no known effect on protein adsorption

Poly(ethylene oxide)[37,75,78]	Structure repeat unit (CH_2- CH_2-O) is able to form a "liquid like" surface with active molecular chains that have no systematic molecular order	In vitro testing has been successful; however, in vivo testing has been inconsistent	PEO and PEG are basically the same polymer, they are just derived from different monomer/polymerization methods
Polyethylene glycol (PEG) [37,75,78]	Very simple structure (structure repeat unit, CH_2- CH_2-O) that forms a hydrophilic surface	Coating is easily influenced by environmental factors	Hydrophilic and nontoxic.
Albumin-coated surfaces [37,75]	Coverage of procoagulant surface. Plasma protein either covalently grafted to the surface or adsorbed onto pre-tethered long aliphatic chains (C8-C18) or PEO -tether warfarin	Similar anti-fouling activity in coated and uncoated grafts	Compared with plasma proteins such as fibrinogen and c-globulin, albumin induces less platelet adhesion This finding prompted its use as an inert thromboresistant coating.
Pyrolytic carbon-coated surfaces[37,75]	Chemical vapor deposition in which hydrocarbon is heated and graphite layer is crystallized renders surfaces hydrophilic	Surface irregularities. Reduced platelet adhesion, yet did not affect overall patency rate of grafts	No information found on leaching/other disadvantages/toxicity. Preclinical studies showed no clear benefit
Phosphorylcholine surfaces[37,75]	Zwitterionic behavior allows it to be electrically neutral at physiologic pH. Is known to limit protein and cell adhesion	Unstable coating; No clear benefit of coating confirmed	
Elastin-inspired coating[37,75]	Limits platelet adhesion and aggregation	Difficult to isolate and purify	
Anti-Inflammatory coatings[37,79]	-Passive strategy to present a nonfouling surface -Active strategy by the active delivery of antiinflammatory agents.	- Passive strategies coatings lack stability as some proteins can displace pre-adsorbed proteins. - The active strategies and other novel approaches (micro and nanoparticles of biodegradable polymers) still require rigorous testing in in vivo models	Active strategies offer many advantages over the passive one. E.g: control over reaction kinetics, highly controlled presentation of immunomodulatory agents, versatility through hybrid systems.

Coatings with Endothelial Cells Attachment[24,76,80]	Endothelial cell receptor modified surface for attachment of cell to create an inert interface.	Larger than ideal scaffold surface microscale topography cell attachment	Increased interest in stem-cell directed endothelialization
Biodegradable polymers (e.g. poly(1,8-octanediol citrate) (POC))[24,81]	Increased hydrophilicity	Biodegradable polymers can influence mechanical properties of devices such as vascular grafts and patches. Porosity may increase with increased degradation rate, increased permeability, and can decreased mechanical stiffness in a nonlinear manner	No effect on graft compliance. Delayed thrombosis in vitro.
Hydrogel and ionic liquid based coatings[75,79]	Highly resistant to protein adsorption	Inconsistent results of their effect on reducing in vivo acute and chronic inflammatory responses	Offer many advantages over traditional strategies, including a viscoelasticity, tunable material characteristics, incorporation of multiple chemical functionalities, nanoscale dimensions with complex architectures, and the ability to deposit onto a variety of material substrates.
Fluoro-Containing coatings[78]	Minimization of surface energy and surface tension, to resist fouling.		

Table 3. Highlighted surface modification approaches and their inferred multi-mechanistic anticoagulation action when they are co-localized on a surface.

Modification with	*Paired Mechanism of Action (MoA)*	*Triple MoA*
Nitric oxide (NO) release 	NO + Zwitterionic  Resistance to nonspecific protein and platelet adsorption and activation/immobilization of biomolecules	
Zwitterionic materials 		
Polyethylene glycol(PEG) 	NO + PEG  Resistance to nonspecific protein and platelet adsorption and activation	NO + Zwitterionic + PEG  Resistance to nonspecific protein adsorption via strong electrostatic and non-electrostatic hydration and antiplatelet/immobilization of biomolecules
	Zwitterionic + PEG  Resistance to nonspecific protein adsorption via strong electrostatic and non-electrostatic hydration/immobilization of biomolecules	
Heparin 	NO + Heparin  Resistance to platelet adsorption and activation and anti-thrombogenic	NO + Zwitterionic + Heparin  Resistance to nonspecific protein and platelet adsorption and activation, anti-thrombogenic/immobilization of biomolecules
	Zwitterionic + Heparin  Resistance to non specific protein adsorption and anti-thrombogenic/immobilization of biomolecules	NO + PEG + Heparin 

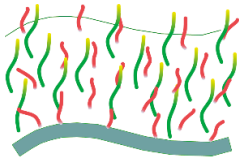
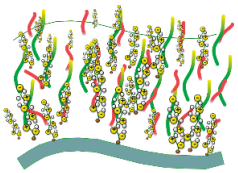
		Resistance to nonspecific protein and platelet adsorption and activation, anti-thrombogenic, and non-thrombogenic
	 PEG + Heparin Resistance to nonspecific protein and anti-thrombogenic	Zwitterionic + PEG + Heparin  Resistance to nonspecific protein adsorption via strong electrostatic and non-electrostatic hydration and antiplatelet/immobilization of biomolecules/antithrombogenic

Table 4: TEG measures of fresh blood and blood stored for up to 3 hours and activated with kaolin or tiffue factor.

	Citrated blood (w/o activation)	**Citrated blood (w kaolin activation)**	**Citrated blood (w TF activation)**
R-time (s)	Time 0 (fresh blood) = 18.5	Time 0 (fresh blood)= 5.6	Time 0 (fresh blood)= 7.9
	Time 60 mins = 12.2	Time 60 mins = 7.4	Time 60 mins = 6.2
	Time 120 min =9.6	Time 120 min =6.5	Time 120 min =6.0
	Time 180 mins = 11.2	Time 180 mins = 6.2	Time 180 mins = 6.0
K-time (min)	Time 0 (fresh blood) = 8.5	Time 0 (fresh blood)= 1.7	Time 0 (fresh blood)= 4.5
	Time 60 mins = 3.3	Time 60 mins = 2.3	Time 60 mins = 2.4
	Time 120 min =3.1	Time 120 min =2.1	Time 120 min =2.4
	Time 180 mins = 3.2	Time 180 mins = 1.9	Time 180 mins = 2.1
α ανγλε (δεγ)	Time 0 (fresh blood)= 28.9	Time 0 (fresh blood)= 66.7	Time 0 (fresh blood)= 42.2
	Time 60 mins = 50.7	Time 60 mins = 60.0	Time 60 mins = 59.0
	Time 120 min =52.2	Time 120 min =61.7	Time 120 min =60.2
	Time 180 mins = 53.1	Time 180 mins = 64.0	Time 180 mins = 62.1
MA (mm)	Time 0 (fresh blood)= 48.9	Time 0 (fresh blood)= 67.6	Time 0 (fresh blood)= 58.0
	Time 60 mins = 55.3	Time 60 mins = 60.6	Time 60 mins = 58.8
	Time 120 min =56.3	Time 120 min =59.4	Time 120 min =58.5
	Time 180 mins = 55.6	Time 180 mins = 59.5	Time 180 mins = 58.2

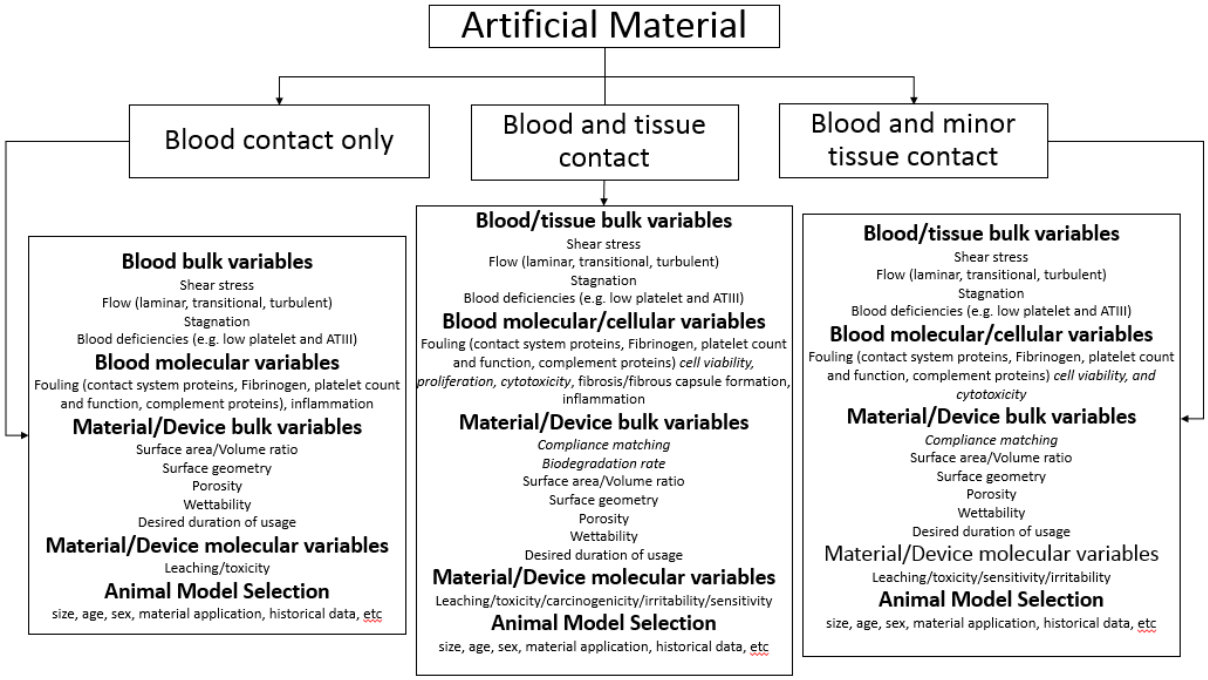


Figure 1: Decision making guideline for pre-clinical hemocompatibility testing of biomaterials

The table of contents

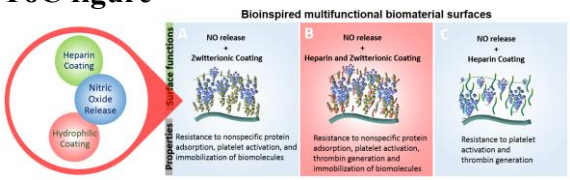
Biomaterials have and continue to play an important role in how we support and treat patients with various diseases through their use in tissue and blood interacting medical devices and drug delivery systems. This review focuses on outstanding challenges and new directions of anti-clotting biomaterials research.

Keywords: anti-clotting self-sterilizing biomaterials

Rana Gbyli, Anna Mercaldi, Harihara Sundaram, and Kagya Amoako*

Title: Achieving Totally Local Anticoagulation on Blood Contacting Devices

ToC figure



ToC figure: Graphical Abstract: An integrative approach to biomaterial surface-focused anticoagulation for implantable and extracorporeal blood-contacting medical devices. Examples of synergy benefits from the combination of bioinspired anti-clotting mechanisms are depicted on surfaces with **a**) nitric oxide release combined with zwitterionic coating, **b**) nitric oxide release with heparin and zwitterionic coatings, and **c**) nitric oxide release with heparin coating.