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Development and hemocompatibility testing of nitric oxide releasing polymers using a rabbit model of thrombogenicity

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Abstract

Hemocompatibility is the goal for any biomaterial contained in extracorporeal life supporting (ECLS) medical devices. The hallmarks for hemocompatibility include nonthrombogenicity, platelet preservation and maintained platelet function. Both *in vitro* and *in vivo* assays testing for compatibility of the blood/biomaterial interface have been used over the last several decades to ascertain if the biomaterial used in medical tubing and devices will require systemic anticoagulation for viability. Over the last 50 years systemic anticoagulation with heparin has been the gold standard in maintaining effective ECLS. However, the biomaterial that maintains effective ECLS without the use of any systemic anticoagulant has remained elusive. In this review, the *in vivo* 4-h rabbit thrombogenicity model genesis will be described with emphasis on biomaterials that may require no systemic anticoagulation for ECLS longevity. These novel biomaterials may improve extracorporeal circulation (ECC) hemocompatibility by preserving near resting physiology of the major blood components, the platelets and monocytes. The rabbit ECC model provides a complete assessment of biomaterial interactions with the intrinsic coagulation players, the circulating platelet and monocytes. This total picture of blood/biomaterial interaction suggests that this rabbit thrombogenicity model could provide a standardization for biomaterial hemocompatibility testing.

Keywords

hemocompatibility; biomaterials; rabbit thrombogenicity model; extracorporeal life support; platelets; monocytes

Introduction

Extracorporeal circulation (ECC) includes a wide variety of devices, from short-term hemodialysis and cardiopulmonary bypass (several hours), to extracorporeal life support (ECLS) (days to weeks).¹ The most serious complications with ECLS devices are bleeding (7–34%) and thrombosis (8–17%).² In a clinical setting, these ECC devices require the use

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of systematic anticoagulation (e.g., heparin) to avoid device thrombosis and failure.³ In addition, the major adverse effect of systemic anticoagulation in ECLS therapy is the risk of bleeding in the patient. Therefore, the desired goal for ECLS therapy is to have devices that are nonthrombogenic and use no or minimal systemic anticoagulation to reduce the bleeding risk. Nonthrombogenic surfaces would promote hemocompatibility of ECC devices and strive to mimic the normal vascular endothelium within these devices. The testing of materials for hemocompatibility is defined with properties that allow contact with flowing blood without causing adverse reactions such as thrombosis, which include platelet activation, hemolysis, complement activation, and/or inflammation. ECLS systems have long strived to use the most hemocompatible biomaterials possible. Therefore, the nonthrombogenic effect and quiescence of circulating blood platelets as blood passes through polymeric tubing used in ECLS has been the elusive goal for hemocompatible polymer research for several years and have been reviewed extensively.^{1, 4-7} This review will focus on the biomaterials that strive to promote platelet quiescence (i.e., nitric oxide (NO) releasing materials) and to evaluate these materials in the human-relevant 4 h rabbit model of thrombogenicity. The challenges in developing a blood/material interface that locally reduces activation of circulating platelets and reduce thrombus formation in ECC devices will be covered in this review as well as developing the animal model that can provide the most informative hemocompatible results.

For ECLS to be successful over the past decades, systemic anticoagulation, specifically with heparin, has been the significant player. This success does have its complications. The major complication, as mentioned above, that occurs with systemic anticoagulation is the high risk of bleeding during ECLS procedures. In an effort to mitigate this risk of systemic anticoagulation, extracorporeal circuits and devices are available with surface heparin coating or coating with other polymers intended to minimize the blood/biomaterial interaction. These modified surfaces may decrease blood surface interaction somewhat, but systemic heparinization is still required when using the surface coatings currently on the market.⁸⁻¹⁰ Table 1 lists the most current material coatings used for mitigating blood/material interactions. Understanding the native local environment at the surface of vascular endothelium can provide the most productive information for the testing of future biomaterials that potentially will not require systemic anticoagulation.

Within the last 20 years of research, materials immobilized with heparin or NO donors have been used as ECLS surface coatings to provide local anticoagulation without the systemic adverse effects.⁸⁵⁻⁸⁸ NO, an endogenous platelet inhibitor and vasodilator, has been incorporated into polymeric tubing to mimic its endogenous inhibitory effects on platelet activation and, thus, attenuate extracorporeal circuit (ECC) thrombus formation. A number of NO releasing (NOrel) polymer coatings have been studied over the last decade and currently the NOrel polymers being studied are polymer-embedded NO donor molecules, such as diazeniumdiolated dibutylhexanediamine (DBHD/N₂O₂)^{85, 89} and *S*-nitrosothiols which includes *S*-nitroso-*N*-acetyl penicillamine (SNAP).^{90, 91} These two polymeric coatings release NO by distinctly different processes but still preserve platelet quiescence and maintain their normal function systemically. These NOrel polymers provide great examples of how the four-parameters of hemocompatibility evaluation (i.e., thrombus

formation, plasma clotting times, platelet count and function) provide a more complete characterization of existing and future nonthrombogenic biomaterials.

Hemocompatibility testing of any biomaterial involves both *in vitro* and *in vivo* assays. However, to date, what specific *in vitro* or *in vivo* assays provide the best information for a complete picture of the biomaterial blood compatibility is lacking. The current standard developed by the International Organization for Standardization (ISO) that guides investigators on testing biomaterials and medical devices for hemocompatibility is ISO 10993-4 which is entitled "Selection of Tests for Interactions with Blood".⁹²⁻⁹⁷ ISO 10993-4 provides a structured test-selection system that is based on clinical concerns which suggests categories of hemocompatibility that should be determined, including thrombosis, coagulation, platelet count and function, hematology, and immunology. Specific tests are available to assess a biomaterial's potential to elicit adverse effects in these categories of blood function with the ultimate goal of ensuring biomaterials and/or medical devices are safe for use in patients. *In vitro* assays available to answer questions of biomaterial and device interactions with blood include both static and circulating blood systems. Static assays include fluorescence-based fibrinogen adsorption or platelet adhesion^{89, 98} in 96-well microtiter plates while circulating blood assays would make use of the Chandler loop system which recirculates blood in a rotating closed loop.^{97, 99-102}

Many *in vivo* animal models have been used to provide a more accurate determination of ECC effects on platelet activation. However, the lack of specific antibodies to platelet P-selectin or cGMP signaling pathway proteins is found in many of these animals such as the sheep or pig. The rabbit, however, lends itself well to these particular antibody evaluations due to strong crossreactivity of the rabbit proteins with human antibodies.¹⁰³ The lower animal cost, the hyper-thrombogenesis and the availability of antibodies for platelet proteins provide a large benefit for using the rabbit.

This review, therefore, promotes the hemocompatible assessment of biomaterials in an affordable and comprehensive *in vivo* rabbit model utilizing a four-parameter configuration which includes thrombus formation, plasma clotting times, platelet count and function determined *via* aggregation, P-selectin expression and cGMP signaling pathway regulation. The utilization of the rabbit thrombogenicity model which includes the four key parameters as a hemocompatibility test for biomaterials and/or medical devices will provide the biomaterial research community with a much needed benefit to predict applicability for clinical utilization. In addition, this model proposes to provide mechanistic understanding of various polymers in the striving to reach hemocompatibility.

Challenges in testing of NOReI polymer/blood interactions using the rabbit thrombogenicity model

The road to the current state of art with regards to the rabbit thrombogenicity model has always worked on the overarching goal that blood circulating through ECLS circuits maintains similar properties that it has normally in the systemic circulation. The challenge to this goal is to first understand the properties of the normal vascular endothelium in

preventing thrombosis and then mimicking those properties into biomaterial coatings of the blood/material interface.

In order to understand the multiple aspects intertwined in the interaction of various biomaterials with circulating blood one needs to first understand normal hemostasis and the possible interaction points. To present an overview of hemostasis is beyond the scope of this review but many superb overviews on the subject exist.^{104–106} However, to provide the reader a working understanding of hemostasis under physiologic conditions and the unregulated hemostatic stimulus that brings about thrombosis to protect against blood loss, the key hemostatic components, coagulation and platelet activation will be summarized. As shown in Figure 1, hemostasis, which maintains blood in a fluid state under physiologic unstimulated conditions, can develop a hemostatic plug when stimulated either through a defect in the vessel wall (extrinsic pathway) or contact activation of the coagulation (intrinsic pathway). Therefore, hemostasis is composed of both the contributions of coagulation and platelet activation pathways when stimulated. When referring to the interaction of biomaterial surfaces with blood, one is specifically describing the intrinsic coagulation pathway contribution and the platelet contribution as the stimulus that inhibits physiological hemostasis and initiates the formation of the fibrin plug with aggregated platelets, red blood cells and leukocytes. Short of lining extracorporeal life support (ECLS) circuits with normal functioning endothelial cells which maintains physiological hemostasis, and which some research is investigating,^{107–110} one is left with developing biomaterials that mimic normal endothelium and reduce the stimulus of contact activation of the intrinsic coagulation pathway as well as activating circulating platelets.

Normal endothelium maintains physiologic hemostasis and the fluid state of blood by inhibiting coagulation, inhibiting platelet activation and promoting fibrinolysis (Figure 1). On the platelet contribution in hemostasis the endothelium releases inhibitors such as prostacyclin, adenosine diphosphatases (ADPases) and NO. With respect to the contribution of coagulation the endothelium inhibits this pathway by secreting heparan which binds to antithrombin III and then to thrombin and inhibits thrombin activity. Exogenous heparin also acts as an anticoagulant *via* the same mechanism and has become indispensable in ECLS therapies (Figure 1). The normal biological response of blood to biomaterials without systemic anticoagulation is illustrated in Figure 2. The initial interaction of blood to a material surface is the adsorption of plasma proteins and in particular fibrinogen. The adsorbed fibrinogen becomes denatured from its native conformation which leads to exposure of the fibrinogen receptor binding sites and the subsequent adhesion of activated platelets. The activated platelets that bind to the surface fibrinogen release thrombin and form a fibrin matrix on the material surface. This complex of fibrin matrix and adhered platelets continue to ‘filter’ red blood cells (RBC) and leukocytes which are also becoming activated. To prevent this clot from forming investigators have strove to find ways to passivate the ECLS circuits. Over the past decade much research has investigated polymer additives that can slowly release physiological relevant levels of NO locally into the flowing bloodstream of the ECLS circuit.^{89, 111–119} Up to 4-h of blood exposure in an *in vivo* arteriovenous shunt model using rabbits was shown in these various studies that local NO flux from the material surface was between 5 and 15 $\mu\text{moles}/\text{cm}^2/\text{min}$, while normal

endothelial cells have a NO flux ranging from 0.5 to 4 $\mu\text{moles}/\text{cm}^2/\text{min}$.¹²⁰ This level of NO flux was shown to be nonthrombogenic in the ECLS circuits. The details about this model and NO fluxes from various biomaterials will be discussed later in this review. The source of endothelial cell NO is illustrated in Figure 3A. *De novo* synthesis of NO is constitutive in the endothelial cell under the regulation of the catalytic enzyme, constitutive NO synthase or cNOS on the substrate, L-arginine. This enzymatic production of NO is dependent upon available calcium. The formed NO then diffuses to underlying smooth muscle cells eliciting vasodilation and to circulating platelets to maintain platelet quiescence.^{115, 121–124} NO released by NO donor-embedded polymers also mediates platelet quiescence similar to that observed with endothelial NO release¹¹⁵ and shown in Figure 3B. Evaluation of the interactions between blood and biomaterials must include parameters that describe how the material stimulates fibrin clot formation and platelet activation and to what degree the materials can reduce their stimulating effect on these parameters.

A goal in studying interactions between biomaterials and blood is to understand the events that lead to contact activation of blood components with the biomaterials. Hemocompatibility testing methods are classified as *in vitro*, *in vivo* and clinical for evaluating the blood-biomaterial interactions. While characterization of biomaterials can be assayed for the most part using *in vitro* methods, the limitations for applied utility in clinical setting are more pronounced without proper *in vivo* evaluations in animal models. Therefore, an integrated approach to biomaterial testing for hemocompatibility will provide the best characterization for the material's interaction with blood. The standards in the United States exist that chemically, mechanically, and physically describe a biomaterial and are based on consensus testing methods from the American Society for Testing and Materials Committee (ASTM).¹²⁵ These standards are for the most part 'voluntary' and not mandatory thus leaving a large testing method interpretation to the individual. It is proposed that *in vitro* whole blood testing methods should be used judiciously in that anticoagulated whole blood which contains living cellular components no longer have oxygenation, nor an energy source (i.e., glucose), are in a highly activated state and becoming necrotic. Therefore, *in vitro* assays using circulating whole blood may have a totally different data interpretation than data from an *in vivo* animal model. With that in mind the *in vitro* assays that may help determine if a biomaterial may provide hemocompatibility are illustrated in Figure 4. The first assay characterizes the material interaction with exogenous human fibrinogen (Figure 4A). In this assay human fibrinogen without any other plasma proteins is evaluated for its ability to adsorb on the biomaterial surface. The second and equally important *in vitro* assay is based on the fibrinogen adsorption assay but includes now the adhesion of fluorescently-labeled platelets to the fibrinogen-adsorbed biomaterial. These *in vitro* assays work together to help determine a biomaterial's ability to adsorb proteins and then the adhesion of activated platelets. If a biomaterial such as any NOrel polymer may show significant fibrinogen adsorption, but because of its antiplatelet activity, can markedly reduce platelet adhesion to fibrinogen-adsorbed NOrel biomaterial (Figure 4B). These two *in vitro* assays can provide a strong starting point for understanding the blood/material interaction observed in an *in vivo* animal model and, in particular, the highly thrombogenic rabbit A-V shunt model.

Current state of NOReI polymers using the rabbit thrombogenicity model

In vivo preclinical testing of potentially new biomaterial polymers in an extracorporeal circuit (ECC) is essential in determining its interaction with flowing blood. However, this testing poses complex interactive processes between the polymer and all components of blood. An *in vivo* model that can for the most part tackle these complex interactions would provide a great opportunity. The interactions that should be evaluated in an animal model would include thrombus formation in ECC, prothrombin (PT), activated partial thromboplastin (aPTT) and thrombin clotting times (TCT), circulating platelet and white blood cell counts, platelet function *via* aggregation and P-selectin activation using flow cytometry and mediation of platelet quiescence through the upregulation of the cGMP signaling pathway. The interpretation of data obtained from these interactive processes will better characterize and define these biomaterials to the clinical situation. One such *in vivo* model that covers many of these blood/biomaterial interaction processes is the rabbit thrombogenicity model used in our laboratory for the past 2 decades.^{89, 111–116, 119, 126–129} The rabbit model can provide laboratories with a more affordable animal model compared with other animals such as the canine, pig or sheep when testing many biomaterials in an arterio-venous (A-V) model.

Even though any warm-blooded animal can be used as an *in vivo* thrombogenic model, an animal that would provide the most complete picture of the blood/biomaterial interaction is preferred. The rabbit along with canine have been shown to have circulating platelets that are more reactive than human, baboon, pig, calf, macaque and sheep platelets to any given biomaterial surface.¹³⁰ As the rabbit and canine platelets being more reactive to artificial surfaces, they would provide the most stringent model for thrombosis. As mentioned above, an *in vivo* thrombogenic model needs to be able to show the interactions of biomaterials on platelet activation through P-selectin upregulation *via* flow cytometry and mediation of platelet quiescence through the upregulation of the NO/cGMP signaling pathway. To investigate these platelet-specific parameters, specific antibodies are needed. Antibodies to these platelet-specific antigens are available in the rabbit unlike other experimental species.^{103, 131} Thus, the rabbit, even with its worst case for promoting thrombogenicity, overall provides a credible species that can evaluate the total interactions as stated above between blood and any biomaterial. The rest of this review will focus on the rabbit as a thrombogenic A-V model (Figure 5) and the interactions between blood and the biomaterials that our laboratory have been investigating for several years, namely the NO releasing (NOReI) polymers.

NOReI polymers have been investigated in our laboratory over 13 years and have seen three generations of NO release polymer development. This NO research has been recently reviewed.¹ As shown in Figure 5, our research used the rabbit A-V shunt as a model for thrombogenicity testing for all surface coatings including NOReI polymers prepared in our laboratory. Recent papers have described the details of the experimental setup for the 4-h studies for biomaterial hemocompatibility testing.^{89, 111–113, 115, 116, 119, 126–128, 132} The key feature of the extracorporeal circuit (ECC) is the larger internal diameter tubing midway in the circuit loop. This larger tubing models the increased surface area and, more importantly, the increased turbulent blood flow that occurs in inline oxygenators and heat exchangers.

Also shown in Figure 5 is a cross-section schematic of the makeup of biomaterial coatings in the medical grade PVC ECC tubing. Each layer is coated by filling tube with tetrahydrofuran (THF)-dissolved polymer such as polyurethanes (PU), decanting solution out of tubing and then a film of surface coatings has worked well for the NOrel polymers and should be satisfactory for any biomaterial coating. The various novel NOrel polymers used for hemocompatibility testing are listed in Table 2. Next, the evaluation of the *in vivo* blood/biomaterial interaction including four end-point parameters, namely, thrombus formation in the ECC thrombogenicity chamber, clotting times of rabbit plasma, degree of platelet function measured by aggregation, flow cytometry and cGMP signaling pathway upregulation due to local NO release from polymer coating will be reviewed.

However, before any discussion of the four end-point parameters a brief description of the rabbit model setup is in order. All animal handling and surgical procedures were approved by the University Committee on the Use and Care of Animals in accordance with university and federal regulations. All rabbits (2.5–3.5 kg) were initially anesthetized with intramuscular injections of xylazine injectable (AnaSed® Lloyd Laboratories, Shenandoah, Iowa) and ketamine hydrochloride (Hospira, Inc., Lake Forest, IL). Maintenance anesthesia was achieved using isoflurane gas inhalation and a small animal ventilator (Engler Engineering Corp., Hialeah, FL). In order to maintain blood pressure stability, IV fluids of Lactated Ringers were given at a rate of 10 ml/kg/h. For monitoring blood pressure and collecting blood samples, the rabbits' right carotid artery was cannulated using a 16-gauge IV angiocatheter (Jelco®, Johnson & Johnson, Cincinnati, OH). Blood pressure and derived heart rate were monitored with a Series 7000 Monitor (Marquette Electronics, Milwaukee, WI). Body temperature was monitored with a rectal probe and maintained at 37°C using a water-jacketed heating blanket. Prior to placement of the arterio-venous (A-V) custom-built extracorporeal circuit (ECC), the rabbit left carotid artery and right external jugular vein were isolated and baseline hemodynamics as well as arterial blood pH, pCO₂, pO₂ and total hemoglobin were measured using an blood-gas analyzer (Radiometer Copenhagen, DK). In addition, baseline blood samples were collected for platelet counts which were measured on a particle counter (Beckman Coulter, Hialeah, FL). Baseline prothrombin, activated partial thromboplastin and thrombin clotting times as well as plasma fibrinogen levels were determined using a coagulation analyzer (Siemens, Deerfield, IL). Baseline platelet function was assessed using an optical aggregometer (Chrono-Log, Havertown, PA) and platelet P-selectin expression were determined utilizing fluorescent-activated cell sorting (FACS) with a flow cytometer (Becton Dickinson, San Jose, CA).

After baseline blood measurements, the A-V custom-built ECC was placed into position by cannulating the left carotid artery for ECC inflow and the right external jugular vein for ECC outflow. The flow through the ECC was started by unclamping the arterial and venous sides of ECC and blood flow in circuit was monitored with an ultrasonic flow probe and flow meter (Transonic HT207, Ithaca, NY). Most importantly, animals were not systemically anticoagulated during the experiments. Rabbit whole blood samples were collected in 3 cc syringes containing 3.2% sodium citrate for cell counts, aggregometry, FACS analysis and western blotting, and 1 cc syringes containing 40 U/ml of sodium heparin (APP Pharmaceuticals, LLC, Schaumburg, IL) for blood-gas analysis. Following the

initiation of ECC blood flow, blood samples were collected every hour for 4 h for *in vitro* measurements. Samples were used within 2-h of collection to avoid any activation of platelets or plasma fibrinogen.

Thrombus formation in the ECC is minimal data gathered in the evaluation of the biomaterial effect in circulating blood. Many investigators have assessed thrombus formation either imaging or, more quantitative, through weighing the formed thrombus.^{89, 111–115, 136, 137} Our modification of these previous thrombus measurements involves still imaging the thrombus in the thrombogenicity chamber of the ECC, but in order to have a more quantitative image National Institutes of Health (NIH, Bethesda, MD) ImageJ software was used to trace the clot and then calculate the area of clot in cm². This area obviously is a 2D representation of the thrombus formed in the chamber after 4-h of circulating blood exposure but on a relative basis is an effective representation of the degree of thrombus formation.^{89, 111, 113, 114} As an example of this imaging process for thrombus area, Figure 6 shows this clearly when comparing the control (no NOrel polymer (A), to the NOrel polymer (B). The 2D images do show that the control thrombogenicity chamber is totally clotted while the NOrel chamber has significantly less clot. After image analysis, the images are quantitated and the thrombus areas plotted (Figure 6C).

The second end-point parameter for blood/biomaterial interaction is the clotting times of plasma during the 4-h ECC experiment. The clotting times which include prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin clotting time (TCT) are evaluated in the rabbit thrombogenicity model to determine if ECLS blood exposure with nonthrombogenic surface biomaterials without systemic anticoagulation preserve a normal range of the three clotting times. However, systemic anticoagulation, namely with heparin, is the norm for ECLS success so, obviously, all clotting times, except PT which includes heparinase, are prolonged. The goal for clotting times when on ECC is to observe no change over the no ECC baseline. Circuits of ECC are known to autoactivate blood components, namely, factor XII (Hageman factor) which initiates the intrinsic coagulation pathway.¹³⁸ Thus, aPTT is the more relevant clotting time for NOrel or any blood-contacting biomaterial. However, all three clotting times include the common pathway of activation of prothrombin to thrombin by activated Xa enzyme and then the conversion of fibrinogen to fibrin. Any loss in any of the enzymes and/or substrates involved in coagulation can lengthen the clotting times. A loss of plasma fibrinogen due to adsorption to ECC surface material can be observed as a longer PT, aPTT or TCT.¹³⁹ As an example of biomaterial surfaces having effects on these clotting times, the NOrel polymer as well as its non-NO containing control polymer both showed increases in PT and aPTT while TCT showed virtually no change in clotting time (Table 3). These increases in PT and aPTT times are probably due to simple hemodilution of the animal's blood fibrinogen and endogenous thrombin as well as loss of plasma fibrinogen due to fibrinogen adsorption to ECC surface. Since this also occurs in the thrombin clotting time measurement, the only difference that explains the no effect of the ECC surface material on TCT is that, in the TCT measurement, thrombin is added exogenously and not hemodiluted maintaining near normal thrombin clotting times. These clotting times are relevant to biomaterials in ECCs by answering an important question about the material possibly promoting any bleeding risk. Dysfibrinogen

or abnormal fibrinogen has been shown to prolong all three clotting times because fibrinogen is an integral part of the common coagulation pathway for PT (extrinsic coagulation), aPTT (intrinsic coagulation) and TCT (common coagulation).^{140, 141} Dysfibrinogen can be acquired and clotting times could be prolonged if the biomaterial surface can directly alter plasma fibrinogen making it abnormal in its formation of fibrin when catalyzed *via* activated thrombin. Understanding a biomaterial's effects on fibrinogen or any coagulation protein is important in future device development to reduce the bleeding liability. Clotting times help in assessing these effects only if no systemic anticoagulation is utilized.

Platelet function and count have become hallmark parameters to measure when ECLS therapy is used clinically.^{142–145} In our continuation of how the rabbit thrombogenicity model can provide a complete hemocompatibility characterization of surface-coated biomaterials, platelet count and function as well as the level of plasma fibrinogen are foremost in this characterization. Platelets are crucial in hemostasis but the normal endothelium within blood vessels strive to maintain platelet quiescence through release of antiplatelet agents such as NO and/or prostacyclin.¹⁴⁶ Two limitations with ECC circuits that strongly activate circulating platelets and reduce platelet count are the adsorption of fibrinogen to the biomaterial surface and the required need for systemic anticoagulation with heparin which has minimal effect on platelet activation or no reduction on fibrinogen adsorption.^{119, 147, 148}

When investigating platelets and their influence on hemostasis in ECCs over hours of time, efforts must be made to maintain circulating platelets in a normal physiological condition as possible. Methods that use animals as source of circulating blood, and not *in vitro* blood reservoirs, provide the best means to study viable platelets as well as other blood components without the detrimental influence of dying cells due to long circulating experiments (up to 8 h) in the *in vitro* scenario. Thus, the rabbit thrombogenic model provides a real situation to maintain blood cells, and particular the platelets, in near normal physiological conditions. Since platelets are important for normal hemostasis,^{149–151} having an evaluation of platelet physiology in this rabbit model or any *in vivo* thrombogenicity model is essential. Three distinct parameters that should be measured to characterize real time changes in platelet physiology when exposed to an ECC should be 1) platelet count or consumption, 2) plasma fibrinogen levels and 3) platelet function as determined *via* aggregation using the optical turbidity method.⁸⁹ ECC circuits have shown over the years of usage that the observed bleeding risk is due to the loss of circulating platelets through artificial surface activation.^{128, 152} Platelet counts can be done by either direct platelet counting from whole blood samples with a hematology analyzer or counting on a particle counter the platelets isolated in plasma rich platelets (PRP). Either method will yield a total platelet count but if platelet function is also important, which we believe it is, then PRP should be prepared. In the rabbit model, PRP was prepared from citrated whole rabbit blood by centrifugation. The PRP sample is normalized to 2×10^8 platelets/ml using platelet poor plasma (PPP) for determining the level of platelet function *via* aggregometry. The other parameter that relates to platelet function is the level of plasma fibrinogen. Plasma fibrinogen determination can utilize the PPP samples used in the aggregation assay.

Fibrinogen levels are measured on a clinical coagulation analyzer. To illustrate how ECCs can influence platelet physiology, Figure 7 shows that NOrel coatings maintain platelet count (A) and preserve aggregation (B). Interestingly, both the control and NOrel polymer ECC show significant loss in plasma fibrinogen over the 4-h experiment (Figure 7C). In case of the NOrel ECC, even though plasma fibrinogen is reduced and may signal a reduction in platelet aggregation these ECCs still preserved platelet counts and importantly function when stimulated. Therefore, tying together platelet count and function with levels of plasma fibrinogen, insights into the polymer characteristics in controlling thrombosis can be ascertained. Another assay for platelet activation that relies upon the technique of flow cytometry is to measure the expression level of platelet surface membrane glycoprotein, P-selectin. The upregulation of P-selectin provides an early biomarker for platelet activation and also can separate ECC effects directly on platelets without any influence of plasma fibrinogen levels. To determine platelet P-selectin (CD62P) and IIb/IIIa (fibrinogen receptor) expressions using flow cytometry, a brief method is described. Whole blood aliquots (100 μ l) were diluted 1:100 with HBSS without CaCl_2 and MgCl_2 and then were directly prepared for cell surface staining of P-selectin and IIb/IIIa using monoclonal antihuman IIIa FITC and monoclonal antihuman CD62P PE antibodies and a flow cytometric analysis. Figure 8 reveals the cytometric analysis used to detect platelets due to size (A), fluorescently detect the expression of P-selectin (B) and then the quantitation of the P-selectin expression comparing unstimulated versus collagen stimulated platelets (C).

Another advantage using a flow cytometric analysis for understanding the effects of biomaterials on platelet physiology is the interaction of platelets after activation with leukocytes specifically circulating monocytes. An illustration of a NOrel polymer eliciting activation of monocytes in the rabbit model is shown in Figure 9. Monocytes are distinguished from other leukocytes by size (Figure 9A) and then activation is measured fluorescently *via* the upregulation of the expression of the surface membrane integrin, CD11b. With this particular polymer, the NO released does show a reduction in the expression of monocyte CD11b (Figure 9B). Briefly, whole blood aliquots (100 μ l) were prepared for cell surface staining of CD11b on monocyte surface membranes with a specific CD11b antibody. Fluorescence intensity of immunostaining of CD11b was quantitated by a histogram log plot analysis after lysis of the red blood cells. Many studies have shown that NOrel polymers as well as other artificial surface/blood interactions can impact leukocyte activity.^{42, 89, 145, 153–158} Therefore, flow cytometric analysis provides investigators early biomarkers for platelet and monocyte activation in characterizing the interaction of biomaterials with circulating blood. A large advantage is that this analysis requires minimal (0.5 ml) blood to gain this information. Flow cytometry as a technique has been in research laboratories for several years,^{89, 113, 159–161} and in most cases instrumentation is readily available.

To illustrate the importance of testing platelet function *via* both aggregometry and flow cytometry analysis, certain biomaterials can show an important discrepancy between platelet aggregation and flow cytometric analysis with respect to P-selection expression. Typically, platelet function determination by aggregation or P-selectin expression can provide similar data about platelet activation. However, in our experience one of our biomaterials, 5 wt% Cu

particle-coated ECC plus an intravenous infusion of the S-nitrosothiol, S-nitroso acetyl penicillamine (SNAP), highlights a discrepancy between reduced platelet aggregation (Figure 10A) and the increased P-selectin expression with exogenous collagen stimulation (Figure 10B). Since platelet aggregation is dependent upon normal fibrinogen for proper aggregation when stimulated with exogenous collagen, the reduction of aggregation 4-h after initiating ECC blood flow would be interpreted that either fibrinogen was not present or dysfunctional or that the platelet itself was unable to respond to collagen. To answer the question that the platelet *per se* was not affected by the Cu-coated ECC plus SNAP IV infusion, flow cytometric analysis was used to reveal that the platelet even after 4-h of ECC blood exposure responded normally to exogenous collagen to upregulate membrane P-selectin (Figure 10B). This showed that this particular biomaterial, with accompanying SNAP infusion, most likely altered plasma fibrinogen in way to block platelet aggregation but not influence the platelet itself in its ability to upregulate P-selectin by exogenous collagen. This ability to answer questions when they arise is an advantage of the rabbit model in that specific antihuman antibodies that crossreact with rabbit P-selectin are available. The availability of specific antibodies for the rabbit suggests the rabbit, and no other animal such as the canine, porcine or sheep, has a strong overall benefit for *in vivo* hemocompatibility testing.

One final note about answering questions dealing with the discrepancy between the loss of rabbit platelets to aggregate and the increased expression of platelet membrane P-selectin is that the available plasma used to determine various clotting times can be used to evaluate the possible changes, due to the biomaterials, of plasma fibrinogen. The configuration of fibrinogen was assessed, for example, with regards to the Cu-coated ECC plus SNAP infusion application *via* Western blotting. Briefly, in the biosynthesis of fibrinogen, the formation of A γ heterodimers linked by disulfide bonds are suggested to be intermediates in the assembly of fibrinogen.¹⁶² To help illustrate the potential of changes in fibrinogen structure Geer et al. recently showed that glutathione and its derivatives including the nitrosated form, S-nitrosoglutathione, interact with several fibrinogen subunits especially the A α subunit, alter its structure and inhibit fibrin polymerization upon activation.¹⁶³ The results from the evaluation of the Cu-coated ECC plus SNAP infusion support Geer et al. results by showing a reduction in the heterodimer A $\alpha\gamma$ formation which is part of the early fibrin polymerization process (Figure 11A and B). This leads to the hypothesis that NO is transnitrosated from the infused SNAP to cysteines in the A α and γ subunits of fibrinogen which explains the attenuated platelet aggregation but no effect on the ability of the platelet to upregulate P-selectin expression.

The last end-point parameter for evaluating blood/biomaterial interactions is determining the level of platelet quiescence and the associated signaling pathways during the 4-h ECC experiment. The question that most investigators of biomaterial/blood interactions are striving to answer is how the biomaterial can best mimic normal vascular endothelium. Biomaterials having properties approaching normal endothelium will ultimately attenuate ECC thrombus formation even without systemic anticoagulation. Platelet quiescence is mediated by two notable endothelium derived inhibitors, namely NO and prostacyclin.¹⁴⁶ The key inhibitor of these agents with respect to preserving the platelet resting state is NO.

Released from vascular endothelial cells, NO regulates the cellular NO/cGMP signaling pathway and the resultant vasodilation in smooth muscle cells^{122, 164} or inhibition of platelet activation.^{165–167} NO mediates these cellular events by directly activating its target enzyme soluble guanylyl cyclase (sGC) and increasing the cytosolic levels of the second messenger cGMP.¹⁶⁸ The intracellular signaling pathway involved in mediating the NO effects on these rabbit platelet responses in this thrombogenic model primarily involves the upregulation of the cGMP-dependent protein kinase, cGKI β , and subsequent elevation of the phosphorylation of its established substrate, the focal adhesion protein, vasodilator-stimulated phosphoprotein (VASP). The phosphorylation of the serine 239 site (P-VASP (ser 239)) of this rabbit platelet intracellular cGMP-dependent protein was mainly upregulated over the other phosphorylation site, serine 157 (P-VASP (ser 157)). Relevance of this P-VASP (ser 239) increase in rabbit platelet upon NO stimulation shows that what is known in human platelet cGMP/cAMP signaling is also present in the rabbit platelet signaling and that the rabbit model can be used to monitor effects of NOrel or, for that matter, any biomaterial polymer in regulating P-VASP (ser 239) and, thus, maintain platelet quiescence. To help review P-VASP expression *via* Western blotting, a brief description of the procedure to isolate and prepare the proper protein lysate from platelets used to investigate the cGMP/NO signaling pathway is in order. PRP was prepared from whole rabbit blood by centrifugation. The PRP was normalized for use in Western blotting studies to determine P-VASP expression levels. Specifically, the upregulation of cGMP protein kinase-dependent VASP (ser 239) phosphorylation after 4-h of blood exposure in a rabbit model of thrombogenicity was observed for NOrel polymers. Figure 12A shows that the NOrel polymer significantly increased in P-VASP (ser 239) expression as indicated on the Western blot by the increased protein band at 47kD over 4-h of ECC blood exposure. The NO released from surface polymers appears to locally maintain platelet integrity and thus mimic the physiological role of NO from the vascular endothelium. The normal endothelium has been shown to release NO at a flux rate between 0.5 and 4×10^{-10} mol cm⁻²min⁻¹ and represents normal local release of NO.⁴⁷ In our work, the NO flux rates for the DBHD/N₂O₂ polymer ECC were initially on average 1×10^{-9} mol cm⁻²min⁻¹, and after 4-h of blood exposure the NO flux rates remained at least 75% of the initial flux rates. Even in face of the strong contact activation of prothrombotic processes elicited by the ECC surface, these ECC NO flux rates maintained ECC patency through marked preservation of platelet quiescence through predominantly the cGMP signaling pathway. Understanding how platelets in this rabbit thrombogenicity model behave to various treatments of antiplatelet interventions such as ECC surface release of NO will provide realistic insight into how these treatments may provide antiplatelet effects in the clinical situation.

Extensive research exists demonstrating that NO mediates the resting state in human platelets by the upregulation of cGMP signaling pathway and increased phosphorylation of both serine 239 and 157 in VASP.^{169–171} The mechanism of NO mediation of platelet quiescence in the rabbit model of thrombogenicity has been investigated using many NO-containing polymers and found that the NO released from these polymer-coated ECCs does preserve platelet quiescence due to upregulation of the P-VASP (ser 239)/cGMP pathway (Figure 12B). The intracellular signaling pathway involved in mediating the NO effects on these rabbit platelet responses in this thrombogenic model primarily involves the

upregulation of the cGMP-dependent protein kinase, cGKI β , and subsequent elevation of the phosphorylation of its established substrate, the focal adhesion protein VASP. The phosphorylation of the serine 239 site (P-VASP (ser 239)) of this rabbit platelet intracellular cGMP-dependent protein was mainly upregulated over the other phosphorylation site, serine 157 (P-VASP (ser 157)). Relevance of this P-VASP (ser 239) increase in rabbit platelet upon NO stimulation shows that what is known in human platelet cGMP/cAMP signaling is also present in the rabbit platelet signaling and that the rabbit model can be used to monitor NOrel polymer effects in mediating P-VASP (ser 239) and maintain platelet quiescence. Previous work using human platelets has demonstrated that VASP and VASP phosphorylation (P-VASP) is a useful biomarker of cGMP signaling and therefore the NO effects in human platelets.^{171, 172} Much of this research has established P-VASP involvement in the inhibition of agonist-induced platelet aggregation and, specifically, the activation of the platelet fibrinogen receptor, integrin $\alpha_{1b}\beta_3$.^{173, 174} Figure 12C hypothesizes that NO released from NOrel polymers coated in ECCs mediates the increase in platelet cytoplasmic cGMP levels, upregulating P-VASP and preserving platelet quiescence. Based on NOrel polymers and effects on P-VASP expression, using the parameter of platelet quiescence mechanism for hemocompatibility testing in the rabbit thrombogenicity model can provide investigators crucial information if their new polymer, NO based or not, can maintain platelets in a resting state *via* upregulation of cGMP or cAMP pathways.

Conclusions and future direction

This review has strived to provide evidence that the rabbit thrombogenicity model can effectively and affordably assess the blood/biomaterial interface. Hemocompatibility is a complex, elusive property that all blood-contacting biomaterials are attempting to gain. The state of the art in blood-contacting biomaterials indicate that locally-released NO from ECC surface polymer coatings can increase hemocompatibility of extracorporeal devices by markedly reducing ECC thrombosis, sustaining relatively normal clotting times, preserving platelet counts and function by maintaining *ex vivo* platelet aggregation processes and upregulating the platelet quiescence mechanism *via* the cGMP signaling pathway and, in particular, the phosphorylation of serine 239 of VASP. Studies investigating improvements in the NOrel polymer to reduce ECC thrombogenicity and medical device commercialization should be part of future studies. The use of the rabbit as a thrombosis model has the potential to provide an effective, affordable monitor of the biomaterial hemocompatibility needed to maintain platelet quiescence and effective ECLS nonthrombogenic surface (Figure 13). This particular model could fill a gap in hemocompatibility testing standardization due to its all-inclusive characterization of biomaterials and their interaction with circulating blood.

Although significant progress has been made in the design and fabrication of hemocompatible polymers in the past years (Table 2), future polymers will need to better approach the function of native vascular endothelium which includes preventing surface-induced thrombosis and maintaining normal hemostasis. Controlling both surface-induced thrombosis and maintaining hemostasis by any one novel biomaterial is a tall order to fill so future biomaterials most likely will work as combination materials that locally attenuate thrombosis and platelet activation but still maintain normal hemostasis systemically. The

future of the rabbit thrombogenicity model will also need to develop into more clinically applicable ECLS by testing hemocompatibility of standard extracorporeal membrane oxygenation (ECMO) or cardiopulmonary bypass circuits which include membrane oxygenators and either roller or centrifugal blood pumps. Many investigational studies are needed to understand how each of these ECLS components, beyond the tubing, can influence the blood/biomaterial interface in a more hemocompatible manner. The important challenge for ECLS without systemic anticoagulation in the future is once a short term or 4-h hemocompatible biomaterial is found then having that hemocompatibility of the ECLS last for a longer term (i.e., up to 30 days for some ECMO patients). The rabbit model provides an ideal forum for pursuing all these future investigational studies.

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Figure 1.

Overview of hemostasis. Hemostasis for maintaining a thrombotic environment when blood comes into contact with foreign surfaces such as extracorporeal life support (ECLS) circuits consists of two contributions. One is the intrinsic coagulation pathway (right) that initiates with factor XII activation and ending with the formation of a fibrin clot. The second contribution is the activation of blood cell components particularly the circulating platelets (left). Both contribution pathways converge at a point of thrombin formation which forms the beginning of the common thrombotic pathway and ultimate hemostatic plug which consists of the fibrin clot and aggregated platelets. As the hemostatic plug matures other blood cell components such as red blood cells (RBC) and activated white blood cells (WBC) also bind in the plug. Fibrinolysis of the plug in the ECLS circuit may not be as important of a process as it is in vessel wounds and healing (i.e., extrinsic coagulation pathway; not shown). The 'a' following the various coagulation factors (right) means activated form of factor. On the left side of hemostasis, vWF is von Willebrand factor, epi= epinephrine, ADP= adenosine diphosphate, TXA₂= thromboxane A₂, PGI₂= prostacyclin and ADPases= adenosine diphosphatases. Two inhibitors of thrombosis are shown on schematic with heparin used to block fibrin formation and endothelial-derived nitric oxide (NO) which inhibits platelet activation.

Figure 2.

Biological response to materials in blood is a multi-step process. Protein adsorption particularly fibrinogen binds to artificial surface and becomes denatured. The circulating platelets are now exposed to the platelet binding site of the denatured fibrinogen and attach to the protein on surface. The binding of platelets to protein promotes their activation and the release of prothrombotic agents namely thrombin. This then ultimately leads to the formation of the mature hemostatic plug consisting of fibrin, aggregated platelets, WBCs and RBCs.

Figure 3.

How biomaterials that release NO can mimic vascular endothelium release of NO. (A) De novo synthesis of NO is constitutive in the endothelial cell under the regulation of the catalytic enzyme, constitutive NO synthase or cNOS on the substrate, L-arginine. The formed NO then diffuses to underlying smooth muscle cells eliciting vasodilation and to circulating platelets to maintain platelet quiescence. (B) Schematic of the NOrel polymer illustrating the release of NO into the lumen of ECC tube to locally reduce platelet activation. This is similar in function as to the normal vascular endothelium. L-arg= L-arginine; cNOS= constitutive NO synthase; iNOS= induced NO synthase; R= receptor; GC= guanylyl cyclase; cGMP= cyclic guanosine monophosphate.

Figure 4.

In Vitro assays to test hemocompatibility. (A) In this fibrinogen adsorption assay, 3000 µl/ml human fibrinogen without any other plasma proteins is evaluated in its ability to adsorb on the biomaterial surface. (B) Platelet adhesion assay uses 3000 µl/ml human fibrinogen but, in addition, utilizes fluorescently-labeled rabbit platelets to determine effect of biomaterials on platelet adhesion. If a biomaterial such as a NORel material shows significant fibrinogen adsorption, but because of its antiplatelet activity, can markedly reduce platelet adhesion to fibrinogen-adsorbed NORel biomaterial.

Figure 5.

In vivo rabbit thrombogenicity model consists of an arterial-venous (A-V) shunt and thrombogenicity chamber. Schematic for testing extracorporeal circuits (ECC) coated with various nonthrombogenic polymers such as the NOrel polymers is shown. Also shown is a cross-section of the ECC tubing polymer coatings to promote ECC nonthrombogenicity. The layers of coatings contain an active layer consisting of the potential nonthrombogenic biomaterial. One example of a biomaterial is one that contained a lipophilic diazeniumdiolated NO donor, dibutylhexanediamine (DBHD/N₂O₂), 10 wt% 1–2 month PLGA and incorporated at 25 wt % into Elast-Eon (E2As) polyurethane. PU= any polyurethane and PVC= polyvinyl chloride.

Figure 6.

Biomaterial effects on ECC thrombus formation in the rabbit thrombogenicity model. Two-dimensional representation of thrombus formation in NORel polymer (NORel) and control polymer ECCs after 4-h blood exposure. (A) Colorized image of thrombus in 3/8 inch I.D. tubing (red region) in the control ECC. (B) Colorized image of thrombus in 3/8 inch I.D. tubing (red region) in the NORel polymer ECC. (C) Quantitation of thrombus area as calculated using ImageJ software from NIH. The data are means $\pm$ SEM. * $p < 0.05$, control ECC vs 25 NORel ECC after 4-h. Reprinted from ref. 16 with permission from Elsevier.

Figure 7.

Biomaterial-coated ECC and its effects on platelet count and function in rabbit thrombogenicity model. Time-dependent effects of NOReI polymer ECC on rabbit platelet count (i.e., consumption) (A), platelet function as measured *via* aggregometry (B) and plasma fibrinogen levels (C) after 4-h of blood exposure in rabbit thrombogenicity model. Data is the mean $\pm$ SEM. * $p < 0.05$, baseline vs control and NOReI ECC. # $p < 0.05$, control ECC vs NOReI ECC. Reprinted from ref. 16 with permission from Elsevier.

Figure 8.

Flow cytometry: evaluation of early platelet activation by biomaterials. fluorescent-activated cell sorting (FACS) analysis for circulating platelets after 4-h blood exposure with NORel or control polymer-coated ECC. (A), Representative FACS analysis graph for unstained circulating platelets. FSC = forward scatter and SSC = side scatter (B) Histogram of P-selectin (CD62P) platelet expression using mouse anti-human CD62P PE antibody conjugate (baseline, no ECC, red filled area). Blue filled area is P-selectin mean fluorescence intensity (MFI) after 40 mg/ml collagen stimulation while the green line denotes the isotype control for P-selectin. (C). Platelet P-selectin mean fluorescence intensity (MFI) after 4-h on ECC with or without 40 ug/ml collagen stimulation in NORel and control polymers. The data are means $\pm$ SEM. * $p < 0.05$, unstimulated vs collagen-stimulated for baseline, control ECC and NORel ECC. # $p < 0.05$, collagen-stimulated for baseline vs collagen-stimulated for control ECC and collagen-stimulated for NORel ECC. The specific MFI data is after the isotype control value was subtracted from each P-selectin MFI value. All FACS analyses used the gated FSC/SSC plot for platelets and 100 μ l of diluted whole rabbit blood (1:100) was used for each determination. Reprinted from ref. 16 with permission from Elsevier.

Figure 9.

Flow cytometry: evaluation of early monocyte activation by biomaterials. Fluorescent-activated cell sorting (FACS) analysis for circulating monocytes after 4-h blood exposure with NORel or control polymer-coated ECC. (A), Representative FACS analysis graph for unstained circulating monocytes. FSC = forward scatter and SSC = side scatter. (B) Monocyte CD11b mean fluorescence intensity (MFI) after 4-h on ECC in NORel and control polymers. (C) Monocyte CD14 (monocyte-specific marker) mean fluorescence intensity (MFI) after 4-h on ECC in NORel and control polymers. The data are means $\pm$ SEM. * $p < 0.05$, baseline vs control ECC or NORel ECC. The specific MFI data is after the isotype control value was subtracted from each CD11b or CD14 MFI value. All FACS analyses used the gated FSC/SSC plot for monocytes and 100 μ l of whole rabbit blood was used for each determination. Reprinted from ref. 16 with permission from Elsevier.

Figure 10.

Discrepancy between platelet activation and ability to aggregate. Platelet aggregation (A) and fluorescent-activated cell sorting (FACS) analysis for circulating platelets (B) after 4-h blood exposure with 10 wt.% Cu⁰-nanoparticle NO generating (NOGen) ± exogenous SNAP or control polymer-coated ECCs plus exogenous SNAP. The data are means ± SEM. *p < 0.05, baseline vs 10 wt.% Cu⁰-nanoparticle NOGen ± exogenous SNAP or control polymer-coated ECCs plus exogenous SNAP. All FACS analyses used the gated FSC/SSC plot for platelets and 100 µl of diluted whole rabbit blood (1:100) was used for each FACS determination. Reprinted from ref. 40 with permission from Elsevier.

Figure 11.

Transnitrosation of fibrinogen could explain the reduced platelet aggregation seen with 10 wt.% Cu⁰-nanoparticle NO generating (NOGen) ± exogenous SNAP in the rabbit thrombogenicity model. Western immunoblot analysis of rabbit plasma fibrinogen after 4-h blood exposure with 10 wt.% Cu⁰-nanoparticle NOGen ± exogenous SNAP or control polymer-coated ECCs plus exogenous SNAP. (A), Representative immunoblot. Aliquots (0.2 ml) of reduced plasma samples after 4-h on ECCs in NOGen or control polymers were separated on 7.5% polyacrylamide gels, protein transferred to poly(vinyl-difluoride) (PVDF) membranes and then immunostained with FITC conjugated goat anti-human fibrinogen antibody. Human plasma fibrinogen was used to confirmed cross-reactivity of the anti-human antibody to rabbit plasma fibrinogen. FGN = fibrinogen. Molecular weight markers are shown on left with positions of the relevant fibrinogen chains and factor XIII indicated on the right. (B) Western blot of plasma samples in (A), after 4-h on ECC in NOGen and control polymers, were quantified by densitometry using Quantity One imaging software (Bio-Rad Hercules, CA). The specific Aαγ dimer formation was calculated as the ratio (density) of the Aαγ dimer band over the sum of dimers plus monomers. The data are means ± SEM. *p < 0.05, baseline vs control ECC/SNAP or 10 wt.% Cu particle NOGen ECCs/_SNAP. Reprinted from ref. 40 with permission from Elsevier.

Figure 12.

Mechanism of NO released from polymer is to increase platelet cGMP and P-VASP which mediate platelet quiescence in the rabbit thrombogenicity model. Western immunoblot analysis of rabbit platelet lysates after 1, 2, 3 and 4-h blood exposure with NOrel or control polymer-coated ECCs. NO effects on P-VASP (ser 239), cGK1β and VASP expressions from rabbit platelet lysates after 2, 3 and 4-h blood exposure with NOrel and control polymer ECCs. (A) Representative immunoblot: After SDS/PAGE electrophoresis and electroblotted blots were immunostained with FITC conjugated mouse anti-human P-VASP (ser 239), goat antihuman cGK1β (L-16) and rabbit anti-human VASP (H-90) antibodies. Molecular weight markers are shown on left with positions of the relevant P-VASP (ser 239), cGK1β and VASP proteins. (B) Western blot of lysate samples in (A) were quantified by multi-channel densitometry using a BioRad Versadoc 4000 digital imager. The ratio of P-VASP (ser 239) over unphosphorylated VASP expressions was calculated. The data are means ± SEM. *p < 0.05, baseline vs baseline + sodium nitroprusside (SNP), 2, 3 and 4-h DBHD/N2O2 containing ECC ± SNP. (C) Schematic of proposed NO mediation of cGMP and P-VASP upregulation that promotes platelet quiescence. ADP= adenosine diphosphate, sGC= soluble guanylate cyclase, SNP= sodium nitroprusside, cGK1β = protein kinase G1 β. Reprinted from ref. 42 with permission from Elsevier.

Figure 13.

Proposed hemocompatibility testing that is effective and affordable using the rabbit thrombogenicity model. Hemocompatibility of a new biomaterial needs to be assessed in the most affordable but also in the most complete evaluation possible to provide the safest and effective result in the clinical setting. *In vitro* assays especially with blood cell components such as platelets need to be tested in a timely fashion and the two assays proposed in testing scheme, fibrinogen adsorption and fluorescent-labeled platelet adhesion, are done within 2–4-hours after blood procurement. The rabbit model of thrombogenicity provides a complete picture of hemocompatibility testing by providing biomaterial effects on thrombus formation, clotting times, platelet count and function by evaluating aggregation, P-selectin expression and the protein that is upregulated when platelets are quiescent, P-VASP.

TABLE 1

Current biomaterial coatings

Coatings	Manufacturer	Active Biomaterial	References
X Coating	Terumo	Poly(2-methoxyethyl acrylate) (PMEA)	11–13
SmarxT	Cobe/Sorin	Alternating hydrophilic/hydrophobic copolymer	14–17
Bioline	Marquet	Albumin + Heparin	18–49
Safeline	Marquet	Albumin	37, 42
Carmeda	Medtronic	Heparin	37, 46, 50–81
Trillium	Medtronic	Polyethylene oxide (PEO) + Heparin	37, 40, 51, 55, 59, 82–84

TABLE 2

Novel biomaterial coatings

Coatings	Active Biomaterial	References
DBHD/N ₂ O ₂ ^a	Nitric Oxide (NO)	118, 133, 134
Cu ²⁺ particles & SNAP ^b infusion	NO	113, 135
SNAP (immobilized)	NO	114

^a DBHD/N₂O₂ = Diazeniumdiolated (D)i(B)utyl(H)exane(D)iamine

^b SNAP = S-nitrosoacetyl penicillamine

TABLE 3

Clotting times (s) 4 h after ECC blood exposure.

Biomaterial	Prothrombin (PT)	Activated Partial Thromboplastin (aPTT)	Thrombin Clotting (TCT)
Baseline	10.2 ± 0.2 (9)	16.7 ± 0.7 (7)	15.0 ± 0.2 (7)
Control	10.8 ± 0.4 (3)	24.5 ± 1.9* (3)	12.0 ± 0.7* (3)
NORel	11.6 ± 0.2* (5)	21.7 ± 1.1* (5)	13.2 ± 0.5* (3)

*
p < 0.05; baseline vs. control or NORel