In Vitro Thrombogenicity Testing of Biomaterials

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The short- and long-term thrombogenicity of implant materials is still unpredictable, which is a significant challenge for the treatment of cardiovascular diseases. A knowledge-based approach for implementing biofunctions in materials requires a detailed understanding of the medical device in the biological system. In particular, the interplay between material and blood components/cells as well as standardized and commonly acknowledged in vitro test methods allowing a reproducible categorization of the material thrombogenicity requires further attention. Here, the status of in vitro thrombogenicity testing methods for biomaterials is reviewed, particularly taking in view the preparation of test materials and references, the selection and characterization of donors and blood samples, the prerequisites for reproducible approaches and applied test systems. Recent joint approaches in finding common standards for a reproducible testing are summarized and perspectives for a more disease oriented in vitro thrombogenicity testing are discussed.

1. Introduction

Cardiovascular disease (CVD) is the number one cause of death globally, and WHO estimated that by 2030, 23 million people will succumb annually to cardiovascular-related diseases.[1]

Beyond medical therapy, interventional procedures are available to treat CVD, which may require implantation of devices such as: vascular prostheses, stents, artificial heart valves, left atrial appendage, or septal occluders (Figure 1). These implants require specific sets of functionalities to gain their therapeutical effect. In case of vascular prosthesis or stents, one functionality is recreating and maintaining the luminal diameter. Further functions represent the hemocompatibility of the luminal surface to avoid thrombotic processes, the histocompatibility to avoid inflammation in the surrounding tissue and degradability of the applied implant-material to reach full regeneration of the vascular wall. The fundamental knowledge about structure-function relationships, with regard to the mechanics already allows targeted design of the structural implant function. Other functions like the blood compatibility are still challenging to integrate in material systems, since the very complex and interlinked interactions at the blood–material interphase are not well understood.

At blood-contacting surfaces, different undesired pathophysiological processes are described: plasma protein adsorption or without conformational change,[2–4] platelet and leukocyte activation, adhesion and aggregation,[5,6] complement or contact activation,[7] and plasmatic coagulation (Figure 2).[8–10] These processes culminate in thrombosis, which, in combination with thrombo-embolization, remain the most serious adverse events and most challenging aspects in the development of novel blood contacting devices.[11–15]

Since clinically applied implant-materials are not sufficiently blood compatible and in many cases exhibit a thrombogenic potential, dual or triple drug therapies with antiplatelet agents and/or anticoagulants are in clinical use to prevent thromboembolic events.[16,17] However, complications such as thrombocytopenia, neutropenia, or haemorrhage occur and even under medication the long-term function of cardiovascular implants is not assured, as reports about stent restenoses and graft occlusions indicate.[18,19] Therefore, multi-functional materials with minimized thrombogenicity are still a clinical need.

The assessment of the material and—at a later stage of development—the device function “(anti-)thrombogenicity” requires the selection of different test methods, which have to be accurate,
reliable, and reproducible.[20–22] This is, not least, important for realizing interstudy comparisons of the thrombogenicity of different materials. According, e.g., to the regulation (EU) 2017/745 of the European parliament and of the council on medical devices, in vitro and in vivo tests are a mandatory parts within the preclinical product verification and validation (see Annex II: Technical documentation, 6.1 Preclinical and clinical data).[23] Beyond the results of these tests, also detailed information concerning the test design, respective protocols and data analysis are obligatory to meet the requirements of the regulation, particularly those formulated in the ISO standard 10993 Part 4.[24,25] Since the regulatory agencies provide recommendations rather than protocols or standard operation procedures, accepted standardizations regarding the preanalytical handling of the blood, test conditions, test parameters, reference materials, static or dynamic conditions, flow conditions, and finally, which type of cells and donors should be included is lacking. Also, standard operating procedures how the testing should be performed are lacking.[8]

This has led to a situation that laboratories perform in vitro assays under substantially different test conditions, including the preanalytical characterization of blood samples, the application of blood from different species, varying anticoagulation and stabilization or storage times as well as test setups. Taking these variations into account, interlaboratory or interstudy comparisons are impossible.[8,26–28]

Here, we review described test methods and procedures for the assessment of the thrombogenicity of materials, prerequisites for a reproducible in vitro testing, applied test system and test parameters for the characterization of the interaction of blood components/cells and the implant. We further review and outline recent approaches that may support realizing reliable, reproducible, and laboratory independent tests.

2. In Vitro Testing According to the ISO 10993-4

Within the ISO norm 10993-Part 4 (Biological evaluation of medical devices, selection of tests for interactions with blood) requirements for the hemocompatibility testing of materials designated for blood contacting applications are depicted for the categories: coagulation, haematology, immunology, platelets, and thrombosis (Table 1).[29,30] Based on these standards, a variety of in vitro tests have been established and are applied, which allow categorization of the thrombogenicity of materials in each laboratory.[20,31] Typically, systematic studies are conducted by the different laboratories to characterize the respective and in vitro test system or to analyze specific aspects of the test protocol such as storage-induced changes in blood cell function and the importance of the application of fresh human blood.[13–15] Furthermore, the influence of the materials applied for the test system, the test duration and flow conditions on the test sensitivity have been studied.[15–17] Other aspects, such as the function of anticoagulants have been reviewed but the selection of anticoagulant (including the grade) and particularly the dosage differ substantially between the working groups.[31,38] Such systematic but non-concerted approaches support the understanding on how to establish reliable and accurate in vitro test systems. However, in order to find common standards and to reach interlaboratory/interstudy comparability and reproducibility, multi-center approaches—similar to those conducted in clinical chemistry—need to be carried out.
A first call for more harmonized and sensitive approaches in the evaluation of the blood/material interactions was formulated by the United States National Heart, Lung, and Blood Institute.\cite{39,40} Here, silica filler-free poly(dimethyl siloxane) and low-density polyethylene were recommended as reference materials (Figure 3). These efforts resulted further in the publication of guidelines for the physicochemical characterization of biomaterials and the blood–material interactions.\cite{40} A later initiative was formulated in 1990 by the International Union for Pure and Applied Chemistry and comprised three topics: 1) the interactions of materials with blood, 2) biocompatibility and inflammation, and 3) in vivo studies.\cite{41} However, data of these studies remained unpublished. Within the European action EUROBIOMAT—Hemocompatibility, poly(vinyl chloride), polyurethane, poly(dimethyl siloxane), polyethylene and polypropylene were selected as reference materials (Figure 3). The results of the concerted physical, chemical and biological characterization were published in 1992 in the book “The reference materials of the European communities: results of hemocompatibility tests.”\cite{42} Selected materials were applied in a range of following studies.\cite{43–45} However, the majority of the here depicted polymers are not commercially available today. In a very recent double-blind and randomized round robin study, stringent standardization of the applied in vitro test protocol was demonstrated to allow a reproducible assessment of platelet adhesion and activation from fresh human adjusted platelet rich plasma.\cite{46} Scoring of the thrombogenic potential of the tested implant materials poly(dimethyl siloxane), poly(tetrafluoro ethylene), and poly(ethylene terephthalate) was equal for all participating centers, thus providing evidence that harmonized common approaches can result in a laboratory independent scoring of the studied parameters. To realize interstudy comparisons, further efforts are necessary and appropriate standards have to be defined, not just by the regulatory agencies, but also by the scientific community involved in the development and testing of new materials for blood contacting applications.

### 3. Prerequisites for a Reproducible In Vitro Testing

#### 3.1. Preparation of Material Samples

The preparation of materials for medical applications requires the application of endotoxin low or free starting materials as well as working under clean room conditions in order to avoid microbial contamination during the synthesis and formation.\cite{47,48} Bacterial endotoxins can activate platelets (aggregation, fusion, and fragmentation), macrophages and neutrophils, which is followed by the secretion of various soluble signaling molecules including interleukins (1, 6, 8), serotonin, histamine, platelet factors, and the tumor necrosis factor as well as thrombus growth (Figure 4).\cite{49–52} Erythrocyte aggregability and deformability is further affected as well as the endothelial cell function, which is related to septic shock.\cite{53–56} Endotoxins can also activate the arachidonic acid cycle and the complement system.\cite{57–59} The successful implementation of these steps has to be confirmed by the quantification of soluble and material adsorbed endotoxins prior any biological testing. This is of utmost importance for excluding endotoxin-induced interactions with blood cells or components, which could superimpose the influence of the material itself on the biological system.\cite{60,61}
The removal of chemical residues (e.g., monomers, initiators, intermediate products, solvents or leachable substances) through thorough cleaning subsequent to synthesis and processing is a further requirement to ensure the production of materials with low cytotoxicity.\cite{62,63} Prior any biological testing of the sample, an appropriate sterilization technique has to be selected. To exclude that the latter has an influence on the functional properties of the final product, physical and chemical characteristics have to be characterized thoroughly before and after the sterilization.\cite{64-66}
Once these conditions are met, cytotoxicity and genotoxicity of the sterilized material samples can be tested according to the ISO standard 10993 (Biological evaluation of medical devices—Part 3 and 5). These in vitro tests are applied to elaborate the indirect influence of, e.g., leachable substances and degradation products as well as the direct influence of the material bulk and surface on, e.g., the cell viability (apoptosis, necrosis), the integrity of the cell membrane, changes in the metabolic activity and lethal alterations in the genetic information of the cells.[67–69]

### Table 1. Typical in vitro tests and tests recommended by the ISO10993-4-2017.[30]

| Category          | Parameter                                | Method                                      |
|-------------------|------------------------------------------|---------------------------------------------|
| Hemolysis         | Mechanically or by material properties   | Total hemoglobin e.g. CBC devices           |
|                   |                                          | Plasma hemoglobin e.g. Cyanhemoglobin       |
| Thrombosis        | Thrombin generation                      | Thrombin-antithrombin complex ELISA         |
|                   | Prothrombin fragment F1+2               | ELISA                                       |
|                   | Fibrinogen                               | ELISA                                       |
|                   | Fibrin peptide A                         | PTT-Test                                    |
|                   | Intrinsic pathway (FXII)                 | Partial thromboplastin time PTT-Test         |
| Platelets         | Platelet retention                       | Morphological changes Blood cell count      |
|                   |                                          | Microscopy                                  |
|                   | Platelet activation                      | Release of β-TG, PF4, TXB2 ELISA            |
| Haematology       | Complete blood cells                     | Blood cell count                            |
|                   | Leukocyte retention                      | Blood cell count                            |
|                   |                                          | Microscopy                                  |
|                   | Leukocyte activation                     | Release of PMN-Elastase ELISA               |
| Complement system | Terminal complement complex              | SC5b-9 ELISA                                |
|                   | Central complement component            | C3a (optional) ELISA                        |

3.2. Donor Selection and Blood Cell Characterization

The selection of blood from animal species (e.g., pig and sheep) instead of human blood has to be avoided, particularly in view of the species-dependent platelet function.[70–73]

In blood products, such as frozen plasma or platelet concentrates, proteins (e.g., factors V and VIII, or cytokines) and cells (e.g., platelet adhesion and aggregation) undergo functional alterations due to the processing (e.g., cooling and freezing), storage (e.g., storage lesion of cells), and

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**Figure 3.** Structural formulas of reference polymers suggested by the United States National Heart, Lung, and Blood Institute[39,40] and the European action EUROBIOMAT—Hemocompatibility.[42]
stabilization. Thus, the use of blood products should be avoided since it very likely lead to an underestimation of the thrombogenicity of the tested materials. For realizing a sufficiently standardized in vitro testing with functional blood cells and components, the application of fresh human blood—according to the criteria for reference values in laboratory medicine—appears to be most appropriate and mandatory. Characterization of the donors should start prior to the blood withdrawal and include the collection of data about recent medication, special nutrition and extensive physical activity. Participants should be excluded from the study if any of these is reported to affect the hemostatic system such as platelet function inhibitors, diabetic and allergic disorders and respective medication as well as excessive consumption of certain food or sports.

Characterization of the donated blood should comprise whole blood haemogram data, analysis of markers of acute inflammation, e.g., C-reactive protein levels as well as test for platelet function (Table 2). At least six (in ideal cases ten) different donors should be included in each study for considering the interindividual variability. While the inclusion of apparently healthy human subjects into initial screening studies is important for achieving reproducible test results, studies at an advanced material/device development stage should also include in vitro test with blood from the designated patient group. The latter can permit deeper insights on the influence of the disease, related comorbidities and the medication on the performance and thrombogenicity of the tested material/device, as previously shown in a pilot study where blood from patients with coronary artery disease was compared to blood from healthy subjects.

3.3. Anticoagulation

Anticoagulation of the blood samples is crucial for avoiding uncontrolled coagulation during the different steps of the in vitro thrombogenicity test. However, the lack of guidelines that define anticoagulants and concentrations, which balance the inhibition of coagulation processes and of blood cells/components function appropriately, necessitates further discussions and matched approaches in the material science community. Today, two major approaches can be distinguished: 1) following the guidelines for clinical laboratory testing and 2) the use of an anticoagulant (and dosage) according to the application in man. The selection of an anticoagulant is also driven by the general goal of the study—basic research or testing at an advanced stage of device development for a defined application and patient group (local or systemic anticoagulation)—as well as by the aim to maintain the analyte (blood cells or components) appropriately functional. Another criterion that may be considered is whether the selection facilitates comparability and reproducibility with data generated in earlier studies.

The most commonly applied anticoagulants in studies utilizing fresh human blood are trisodium citrate, sodium heparin and hirudin. Each of it has its advantages and disadvantages, which should be considered carefully. Studies focusing on the thrombogenicity evaluation can follow the recommendations for the use of trisodium citrate—a calcium chelator—(standardized concentrations of 0.105–0.109 M and 0.129 M trisodium citrate) as well as the guidelines for preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) in platelet function testing. The binding of calcium by citrate inhibits its function as a cofactor for complement

Figure 4. Platelet adhesion/activation and thrombus formation on a bacterial contaminated polymer surface. A) Adherent platelets, B,C) microthrombi containing platelets stabilized by a fibrin mesh. On all surfaces, platelet derived microparticles are visible (Scanning Electron Microscopy, platelets in orange and bacteria in green pseudocolors). [For detailed information see Supporting Information.]
activation and vitamin K-dependent proteins of the coagulation cascade as well as—to a certain extend—platelet activation. However, earlier studies—conducted in different in vitro test systems—showed that for instance adhesiveness of platelets on materials is greater in citrated than in heparinized blood and that this parameter is not related to residual concentrations of calcium in plasma.\[^{98,99}\] Moreover, the results of a recent German round robin study proved that under appropriate in vitro test protocol standardization and citrate anticoagulation of fresh human blood, scoring of, e.g., platelet adhesion and activation on different polymeric implants was reproducible between the test centers.\[^{46}\]

Table 2. Preanalytical blood parameters for the characterization of apparently healthy humans. The given reference ranges are laboratory and device specific, respectively. (Reprinted from Hemocompatibility of Biomaterials for Clinical Applications. Blood-Biomaterials Interactions, S. Braune, A. Lendlein, F. Jung, Developing standards and test protocols for testing the hemocompatibility of biomaterials, pp. 51–76, Copyright (2018) with permission from Elsevier/Woodhead Publishing).

| Parameter | Unit | Reference ranges |
|-----------|------|------------------|
| Donor data | | |
| Donor number, measurement date | | |
| First name, last name | | |
| Date of birth | | |
| Sex | m/f | |
| Age | years | |
| Height, weight, body mass index | cm, kg | |
| Blood pressure | Low | Slightly | Elevated | Strong |
| Blood pressure systolic | mmHg | <100 | 140–159 | 160–179 | >180 |
| Blood pressure diastolic | mmHg | <60 | 90–99 | 100–109 | >110 |
| Heart rate | 1 min\(^{-1}\) | | | |
| Hemogram | Woman | Men | |
| Leukocytes (WBC) | 1 \(\times\) 10\(^5\) µL\(^{-1}\) | 3.9–10.0 | 4.2–9.1 |
| Erythrocytes (RBC) | 1 \(\times\) 10\(^6\) µL\(^{-1}\) | 3.9–5.2 | 4.6–6.1 |
| Hemoglobin (HGB) | mmol L\(^{-1}\) | 7.5–9.9 | 8.7–11.2 |
| Hematocrit (HCT) | % | 34.1–44.9 | 40.1–51.0 |
| Mean corpuscular volume (MCV) | fl | 79.4–94.8 | 79.0–92.2 |
| Platelets (PLT) | 1 \(\times\) 10\(^3\) µL\(^{-1}\) | 182–369 | 163–337 |
| Mean platelet volume (MPV) | fl | 7–13 | 7–13 |
| Unspecific inflammatory markers | | | |
| C-reactive protein (CRP) | µg mL\(^{-1}\) | \(\leq 5\) = Negative, 5–10 = Suspicious, >10 = Positive |
| Platelet aggregation | | | |
| Maximum induced platelet aggregation (mPA) | | | |
| mPA collagen (10 µg mL\(^{-1}\)) | % | 81–95 |
| mPA ADP (20 \(\times\) 10\(^{-6}\) µ) | % | 79–98 |
| mPA ristocetine 1 mg mL\(^{-1}\)) | % | 72–105 |
| Spontaneous aggregation | % | 1–10 |
| Aggregation speed (vPA) | | | |
| vPA collagen (10 µg mL\(^{-1}\)) | % min\(^{-1}\) | 79–183 |
| vPA ADP (20 \(\times\) 10\(^{-6}\) µ) | % min\(^{-1}\) | 97–164 |
| vPA ristocetine (1 mg mL\(^{-1}\)) | % min\(^{-1}\) | 33–138 |
| Spontaneous aggregation | % min\(^{-1}\) | 1–10 |
| Platelet function analyzer (PFA-100) | | | |
| Closure time collagen/epinephrine | s | 84–160 |
| Closure time collagen/ADP | s | 68–121 |
| Platelet activation | | | |
| CD42a\(^{\text{a}}\)/CD62P\(^{\text{p}}\) platelets | % | \(\leq 5\) |
In whole blood test systems, heparin (an indirect thrombin inhibitor) is frequently applied in accordance to bio-functional considerations and to therapeutic treatments with systemic anticoagulation.\textsuperscript{100–104} Gao et al. have shown that heparins (e.g., unfractionated heparin, low molecular weight heparin, and fondaparinux) have a proactivating effect on platelets through induction of an outside-in signaling via the GPIIb-IIIa complex.\textsuperscript{105} The direct binding of heparin to GPIIb-IIIa, the induction of phosphatidyl-inositol-3 kinase activation and of conformational changes of the receptor complex by heparin could be confirmed also in later studies.\textsuperscript{106} This binding mechanism appears to be independent of whether heparin molecules are circulating or surface bound.\textsuperscript{107} Heparin was also shown to potentiate the effect of low-dose ADP on platelet activation and to increase fibrinogen binding to GPIIb-IIIa.\textsuperscript{105,108} Consequently, the threshold for platelet activation by agonists such as ADP or fibrinogen is lower when heparin is applied.\textsuperscript{109} Another concern is the sensitivity of heparin to PF4, which is released by activated platelets. Platelet-membrane bound heparin-PF4 complexes are prone to the binding to pathogenic antibodies. The latter can induce heparin-induced thrombocytopenia (HIT) and thus can cause platelet activation, aggregation and thrombosis.\textsuperscript{110} The use in substantially differing concentrations (0.5–5 IU heparin mL\textsuperscript{-1} whole blood) and functional forms (unfractionated and low molecular weight heparins), however, in many cases does not allow interstudy comparisons. While hirudin as direct thrombin inhibitor primarily finds application in studies on the interactions of material-induced complement activation, it is discussed for hemocompatibility testing in principle, since platelet activation and activation of the initial processes of coagulation also appeared to be less influenced by hirudin than by heparin.\textsuperscript{111–114} Similarly to heparin, a standardization of the concentration is lacking. Due to the ability of ethylene diamine tetra-acetic acid (EDTA, 1.5–2.2 g L\textsuperscript{-1} whole blood) to inhibit platelet aggregation and thrombus formation (via conformational changes in the platelet GPIIb/IIIa receptor) as well as its time-dependent influence on the platelet morphology (particularly the mean platelet volume) it is not recommended for thrombogenicity studies.\textsuperscript{115–117} However, its functionality also leads to the dissolution of reversibly bound platelet aggregates, which can be used for measuring platelet retention on material surfaces by the enumeration of single circulating platelets.

3.4. Collection, Preparation, and Storage of Blood

From the British Committee on Standards in Haematology and the International Society on Thrombosis and Haemostasis guidelines are available describing standardized blood collection protocols with minimal clotting and activation.\textsuperscript{96,97} During the blood withdrawal, e.g., from the vena cubitalis of a resting donor, minimal venostasis and needles with a minimum of 21 gauge should be applied.\textsuperscript{118–120} Withdrawal of the blood should be performed with relatively slow suction and handling of the blood container should be careful in principle. Taken together, these are necessary steps to exclude shear-stress induced activation processes in the fresh blood.\textsuperscript{121} The collected blood and anticoagulant should be homogenized under continuous mild agitation during the procedure, which can be realized, e.g., with winged/butterfly infusion set with associated tubes (siliconized glass or polypropylene). In order to ensure the accurate anticoagulant to blood ratio, the entire filling of the tube is crucial. Despite the necessity of discarding the first 3–4 mL of blood is still under debate, this approach is recommended in order to avoid coagulation activation in the syringe due to tissue factor/thromboplastin release as a consequence of the tissue damage during the venipuncture.\textsuperscript{121–123} A resting phase of 15–30 min subsequent to the blood collection is recommended to ensure that the blood cell function (particularly platelet activation) is not altered by the preparation steps.\textsuperscript{124} Preparation of PRP and PPP should be conducted according to the above mentioned international guidelines haemostasis and thrombosis testing.\textsuperscript{125} It is recommended to very well control the temperature and to reduce thermocycling of blood samples during the processing for avoiding temperature-gradient-induced alterations of blood components and cell activation.\textsuperscript{122,126,127} It is under debate whether keeping the temperature during blood preparation at room temperature (according to the clinical routine) or at 37 °C is more appropriate for reducing the influence of temperature gradients, in particular, since most of the commercially available tests are performed at 37 °C.\textsuperscript{128–132} To minimize the effects of storage time on blood cells and components (cell lesion and proteolytic digestion of proteins) and to ensure an appropriate function of these, all experiments should be completed within 4 h post the blood withdrawal.\textsuperscript{129,133–136}

3.5. In Vitro Test Systems

Various considerations can be decisive in selecting an appropriate test system, such as:

- the format of the material (e.g., (non)porous, particle, film),
- its developmental status (e.g., early candidates, prototype) and
- the kind of intended use (e.g., extracorporeal, implantable device).

Tests mimicking no or low flow (static) conditions are typically used for, e.g., qualitative and quantitative protein adsorption and platelet adhesion/activation studies.\textsuperscript{137,138} Tests under dynamic blood flow conditions differ in their setup, their flow dynamics and blood contacting surfaces for the test system itself.\textsuperscript{139,140} A brief overview about typical setups is given in Figure 5. A dynamic setup may ideally simulate the (patho-)physiological conditions during the application of the device.\textsuperscript{141,142} Addressing the varying responses of blood cells and components to arterial- (high shear, pulsatility), venous- (steady low shear, oscillatory, static), and pathological- (ultra-high shear, recirculation, stagnation) flow conditions is of further relevance in the evaluation of the usability of the test material/device.\textsuperscript{143–145} Short-term and continuous supraphysiologic shear stresses—, e.g., in artificial heart valves, stents, blood pumps and dialysis tubings—can induce conformational changes in blood plasma proteins (e.g., von Willebrand factor), platelet and leukocyte activation (e.g., platelet-leukocyte aggregation) as well as lysis of platelets (release of soluble activators and microparticle formation) and erythrocytes (hemolysis).\textsuperscript{146–153} On the other hand, low shear forces,
recirculation and areas of stagnation—occurring, e.g., in the neo-sinus of transcatheter heart valves and in secondary flow areas of centrifugal blood pumps—can also induce thrombotic processes. Evaluating these processes under appropriate (and varying) shear conditions is particularly relevant when the device reached a certain level of development since hemodynamics and device geometry can influence the blood–material interactions in the near vicinity but also for downstream activation processes.

Further considerations should comprise the begin and duration of the biological processes to be measured as well as the overall test time to ensure an appropriate functionality of the blood proteins and cells during the assay. Further attention should be paid to the influence of test setup components (e.g., thrombogenicity of the blood contacting parts of the test system, air blood interfaces, pump-induced hemolysis) and handling (e.g., storage time of blood) on the functionality of the blood proteins and cells. Rough and porous implant surfaces should be evacuated prior any testing to avoid the formation of prothrombotic microscopic air bubbles during testing. The same care must be taken to avoid the formation of micro air bubbles due to leaks in the test system (e.g., within tubing connectors).

4. Characterization of the Blood–Material Interactions

4.1. Protein Adsorption

The multifaceted interactions of blood with cardiovascular devices are determined by their geometry (inducing, e.g., blood cell trauma), the shear forces present in the device and on the blood contacting surfaces (inducing, e.g., VWF unfolding and platelet activation/aggregation) as well as the physical and chemical properties of the materials used (e.g., available functional groups, surface electrical charge, hydrophilicity/hydrophobicity, linker molecules, interfacial adaptability and surface roughness). In this interplay between the acellular/cellular blood components and the device surface, adsorption of plasma proteins is considered as the initial event and basis for all following interactions.

Vroman et al. described that proteins exhibiting a high mobility arrive earlier and are replaced by those with higher surface affinity (Vroman effect). The composition of the layer of adsorbed proteins is, thus, not static but changing with time and a precise prediction of the protein adsorption and desorption processes still remains challenging. The available reports, particularly for thrombogenic materials, indicate that albumin and immunoglobulins (IgG) precede the adsorption of fibrinogen and fibronectin, which later can be replaced by high molecular weight kininogen or factor XII. Established methods for studying the composition of adsorbed protein layers are spectroscopic and mass spectrometry-based techniques such as high resolution tandem or shotgun mass spectrometry (desorption, digestion, and separation of surface adsorbed proteins followed by the identification of the protein fragments via bioinformatics analysis), spectroscopic ellipsometry (dynamic studies of adsorbed layer growth), X-ray photoelectron spectroscopy (XPS, majorly single component analyses), secondary ion mass spectrometry (SIMS), and time-of-flight (ToF)-SIMS as well as matrix-assisted laser/desorption/ionization (MALDI) time-of-flight mass spectrometry (MALDI-ToF/MS). These techniques allow studying protein adsorption on material surfaces, which were contacted with multicomponent or complex solutions, such as blood plasma. Coupling of these methods allows resolving different proteins of various molecular masses. The combination of MALDI-ToF/MS with techniques that allow further separation of proteins, such as 2D gel electrophoresis, allows resolving single proteins by the mass spectroscopy.
Immunological methods can be considered for quantifying and visualizing adsorbed proteins after contacting material surfaces with single- or multi-component solutions, such as blood plasma or whole blood.[106,107]

Beyond the composition of the protein layer, also the conformations of the adsorbed proteins influence the interactions with blood cell/components and the material surface.[108]

Shear- or adsorption-induced conformational changes can lead to the exposure of binding motifs that are not accessible, to cell receptors and enzymatically active blood components in the native state of the circulating protein.

An example how device geometry can severely influence the hemostasis represents the acquired von Willebrand syndrome, a bleeding disorder that has been reported in patients during left ventricular assist device support.[109] The shear stresses in such devices can lead to an extensive unfolding of VWF and a rapid loss of large VWF multimers either through the cleavage by ADAMTS-13 or through the consumption by platelets (consumptive deficiency).[109] Overall, this is associated with reduced binding capacities of VWF to the subendothelial collagen and an associated risk of bleeding.

Adsorption-induced conformational changes have been shown particularly for fibrinogen, a protein majorly involved in the primary hemostasis. The degree of unfolding and associated expression of platelet binding sites (located at the fibrinogen γ-chain and α-chain) correlate with the activation and adhesion of platelets to material surfaces, while they do not with the amount of adsorbed fibrinogen.[110,111] This seems to represent a fundamental concept since analogous reports are available for proteins, which are not involved in the cascade systems and considered to reduce the thrombogenic potential of device surfaces, such as albumin.[112–114] Similarly to fibrinogen, albumin mediates the adhesion of platelets to material surfaces via RGD-specific receptors if the protein exhibited a certain degree of unfolding.[115–118] To assess the orientation, conformation and bioactivity of surface adsorbed proteins a variety of techniques can be applied such as circular dichroism spectropolarimetry, Fourier transform infrared spectroscopy, surface enhanced Raman scattering, atomic force microscopy as well as quantitative and qualitative immunological techniques—such as fluorescence microscopy and ELISA—can be applied.[116,117]

4.2. Contact Activation

The cascade systems of blood function according to similar principles: recognition molecules target body-foreign structures, which can induce the generation of various mediators. One activation route that can be initiated is the contact system also termed intrinsic coagulation.[118,119]

Factor XII (FXII, Hageman factor)—the main circulating recognition zymogen—was reported to interact with negatively charged surfaces (e.g., cellulose), but also with highly wettable and highly nonwettable surfaces. This results in conformational changes of the protein, which can lead to its nonproteolytic conversion (autoactivation) into the active enzyme α-FXIIa.[120,121] The surface adsorbed α-FXIIa can cleave circulating FXII to enzymatically active β-FXIIa. These processes initiate the kallikrein/kinin pathway (via prekallikrein cleavage and bradykinin generation) or the intrinsic pathway of coagulation (via FXI activation) and converge in thrombin generation and thrombus formation.[122,123] Contact activation can be determined by partial thromboplastin time assays. Chromogenic assay and ELISA are applied to measure FXIIa activity.[124–126]

4.3. Complement System

Activation of the complement cascade—as part of the human innate immune response—is associated with conformational changes of the components involved in the different activation pathways.[127–129] Particularly activation of the alternative pathway was reported for hydrophobic surfaces and materials containing amine or hydroxyl groups.[130,131] These processes are thought to involve the covalent binding of complement component C3 and its conversion into C3b-like molecules as the dominant activation route. Interactions of non-antigen-bound IgG and C1(q) (part via electrostatic and hydrophobic interactions)
with biomaterials surfaces are reported to induce activation of the classical pathway.\textsuperscript{[11–15]} Analysis of plasma levels of C3a and the terminal lytic C5b-9a complex by ELISA represent the most common quantitative measurements for complement activation.\textsuperscript{[209,216,217]} Beyond this, a number of reports indicate the role of further complement components in the material-induced complement activation (e.g., C4) and analyzing these processes directly at the blood–material interphase, e.g., via optical and atomic force microscopy or quartz crystal microbalance approaches.\textsuperscript{[209,211,218–221]}

4.4. Hemolysis

In the context discussed here, hemolysis describes rupturing of red blood cells and subsequent release of intracellular molecules, particularly after contacting implant surfaces. The released molecules can induce platelet activation, e.g., directly via ADP and indirectly through the reduction of NO by the released hemoglobin.\textsuperscript{[222]} It has been shown that red blood cells in such conditions may enhance conversion of prothrombin to thrombin.\textsuperscript{[223]} Similarly, microparticles produced by erythrocyte fragmentation during hemolysis have polynegative nches, which activate blood coagulation by thrombin generation.\textsuperscript{[224]} Plasma hemoglobin and heme as products of erythrocyte destruction during hemolysis stimulate the expression of the adhesion molecules ICAM-1 (intracellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule 1), and E-selectin on endothelial cells, leading to vessel obstruction, tissue hypoxia and ultimately tissue death.

Hemolysis can be induced by:

- substances leaching out of the implant, e.g., metal ions and unreacted monomers\textsuperscript{[225–228]}
- air-blood interfaces\textsuperscript{[229–231]}
- high shear rates and\textsuperscript{[232,233]}
- complement components, e.g., C5a.\textsuperscript{[210,212,234]}

Beyond the erythrocyte count, free hemoglobin in plasma is the classical analyte for hemolysis testing. It can be determined, e.g., by the spectrophotometrical cyanmethemoglobin technique, by hematology analyzers as well newer methods such as oximetry.\textsuperscript{[235,236]} Strategies for the standardization of incubation conditions in hemolysis testing have been reported by Henkelman et al.\textsuperscript{[227]} This work underlines that different recommended test techniques (animal vs human blood, extract vs bulk testing, etc.) can result in a varying categorizations of the samples. They concluded that testing material samples with diluted human blood (or washed human red blood cells) for 24 h at 37 °C resulted in the most sensitive assay.

4.5. Platelets

The thrombogenicity of a material surface is described as one of the major reasons for the failure of blood-contacting medical devices.\textsuperscript{[6,22,237,238]} As depicted in the ISO 10993-4 (Selection of Tests for Interactions of Blood), the examination of material-induced thrombosis is one of the key requirements in the evaluation of new implant materials.\textsuperscript{[306]} Key elements of the blood-biomaterial interactions are platelets, specialized fragments of megakaryocytes that play central roles in the process of thrombus formation.\textsuperscript{[21,219–221]} Thus, the interaction of platelets with the surface of blood contacting biomaterials is the key for understanding the thrombogenicity of a material.\textsuperscript{[95,242]}

The first phase of thrombus formation (tethering) is characterized by the interactions of platelets with surface bound proteins via varying adhesive glycoproteins (GP), such as the GP Ib (~25 000 receptors per platelet, e.g., VWF binding) and the GPIIbIIIa (~600 000–100 000 receptors per platelet, e.g., fibrinogen and VWF binding). This process initiates the activation of platelets (outside-in signaling) and results in the stabilization of the adhesion through the formation of pseudopodia and spreading (second phase).\textsuperscript{[243,244]} During the third phase, adherent platelets recruit circulating ones through the secretion of soluble activating substances such as ADP, P-Selectin (sCD62P) and platelet factors (e.g., PF4), which leads to the formation of a platelet aggregate (thrombus).\textsuperscript{[245]} The fourth phase is characterized by the expression of phospholipid phosphatidyl serine on the platelet membrane, which promotes the activation of the coagulation cascade and results in thrombin generation and fibrin polymerization. This process is initiated within 30 s, leads to the formation of stable thrombi within 3–5 min and can persist for months.\textsuperscript{[246,247]} Thrombus formation can lead to an occlusion of the device and its near vicinity, which, for instance, represents the major reason of graft failure and in-stent neatherosclerosis.\textsuperscript{[248–250]} Thrombi might also detach from the device surface, form emboli and lead to ongoing gangrene or major adverse events such as stroke and pulmonary infarction.\textsuperscript{[251,252]}

The evaluation of the material induced platelet activation on material surfaces and in the circulation as well as the formation of thrombin represent key aspects in understanding the thrombogenic potential (thrombogenicity) of a device surface.\textsuperscript{[21,253]} Analysis of the adhesion and activation of platelets (shape change and spreading, see Figure 7a) can be considered as an initial and robust thrombogenicity tests that can be performed in different screening approaches.\textsuperscript{[31,254,255]} One advantage of performing these assays under quasi-static conditions is that the interaction of platelets or plasma proteins with the material surface can be evaluated excluding, e.g., the activation of platelets by shear forces. Various light or electron microscopy techniques can be applied to study—qualitatively and quantitatively—the adhesion of platelets as well as the formation of occlusive thrombi on the material surface (see Figure 7).\textsuperscript{[160,256–258]} Beyond this, analysis of the nonadherent (circulating) platelets (platelet-platelet- and platelet-leukocyte-aggregates) have been shown to be crucial in the thrombogenic testing, particularly for those materials that do not facilitate platelet adherence but can prime their activation after platelets contacted the material surface (e.g., hydrogels).\textsuperscript{[259–261]} The number of nonadherent cells (retention and consumption), their degree of activation as well as the formation of micro-thrombi and platelet derived microparticles are classically determined.\textsuperscript{[262–264]} Expression of activation markers on the platelet membrane (e.g., P-Selectin) and the release of the soluble agonists from granules and lysosomes (e.g., sP-Selectin, Platelet Factor 4 and β-thromboglobulin) indicate their degree of activation and can be studied via flow cytometry and ELISA.\textsuperscript{[265–268]} In many cases,
dynamic in vitro test systems are applied, which allow repeated
interactions between the circulating blood cells and the ma-
terial surface (see section In vitro test systems). These test sys-
tems are also very well suited for studying material-induced
thrombogenicity time-resolved, which may provide further
insights in the principle mechanisms of thrombotic processes
induced by artificial surfaces. Here, the work of Nesbit et al.
and Stalker et al. on the heterogeneous core and shell like
distribution of platelet activation in thrombi can serve as an
example. Their observations revealed that the thrombi
core covers the close vicinity of the activation site and is char-
acterized by densely packed degranulated P-Selectin expressing
platelets (and thrombin). Platelets in the surrounding shell
are less densely distributed and are expressing the marker to
a lesser extent. These data correlated very well with those of
Merten and Thiagarajan who showed that P-Selectin expression
on platelets determines size and stability of circulating platelet
aggregates. Results of these studies furthermore underline
the importance of correlating activation processes in the circu-
lation (e.g., activation of circulating cells and soluble markers)
with those occurring on the material surfaces.

4.6. Leukocytes

Thrombus growth is associated with a range of interlinked
feedback loops between platelets, the acellular activation path-
ways and leukocytes. A plethora of soluble substances released
from platelet granules after activation (e.g., growth factors
β-thromboglobulin and Platelet Factor 4, various growth fac-
tors and hemostatic proteins) and granule membrane proteins
that are translocated to the platelet surface such as P-Selectin
can lead to the recruitment and activation of leukocytes, e.g.,
via the P-selectin glycoprotein ligand-1 (PSGL-1). Particularly,
monocytes and neutrophils are reported to contribute to
the local thrombogenesis within minutes either through cell-
cell interactions—such as the formation of platelet-leukocyte
aggregates—or with the biomaterial/implant surface —, e.g., via
adsorbed complement components—for a detailed review see
the work of Gorbet and Selton[6] (Figure 2). The degranu-
lation of pro-inflammatory and pro-coagulant substances (prosta-
glandin, bradykinin, leukotrienes, cytokines, etc.) and the release
of oxidative products as well as the expression of tissue factor
(TH-pathway) and CD11b (binding of fibrinogen or FX) on the
leukocyte membrane are associated with the material-induced
leukocyte activation. These inflammatory processes can also
induce activation of the endothelium (e.g., to cells in the near
surrounding the vascular implant) and result in a shift towards
the prothrombotic and proinflammatory phenotype. How-
ever, our understanding of many of these interlinked processes is
still fragmented and subject of a recent research. Among
other aspects, the contribution of neutrophil extracellular trap
formation on thrombus growth is subject of recent research.[280]

Interaction of leukocytes with material surfaces can
be determined directly, e.g., by visualizing adhesion and
activation-induced morphological changes at the material
surface. Counting of nonadherent leukocytes—e.g., via a
complete blood cell count—represents an indirect quan-
tification of the retention of these cells to the material
surface. Leukocyte activation as well as their contribution to
the thrombus formation (platelet-leukocyte aggregates) can be
measured, e.g., by CD11b (Mac-1) labeling (flow cytometric
and microscopic analysis).[281,282] Levels of soluble activation
markers, such as polymorphonuclear granulocyte elastase
(PMN- elastase) or reactive oxygen species (indicating respira-
tory burst), can be quantified by ELISA and also by flow cytom-
etry (e.g., dihydrorhodamine-12 conversion).[283,284]

4.7. Thrombin Generation, Fibrinogen-Fibrin Conversion,
and Fibrinolysis

As key enzyme of the coagulation, the generation of
thrombin can be determined, e.g., indirectly in the form of

Figure 7. Representative microscopic images of adherent blood cells/components with implant material surfaces. Interaction of platelets and
leukocytes with poly(tetrafluoro ethylene) A) and poly(dimethyl siloxane) C) (glutardialdehyde induced fluorescence staining, confocal laser scanning
images). Thrombus formation on metallic and polymeric implant surfaces—B,D) reflected light microscopy images). [For detailed information see
Supporting Information.]
thrombin-antithrombin-complex III and prothrombin fragment F1+2 levels (e.g., ELISA) as well as a measure of its activity (e.g., chromogenic assays). The conversion of fibrinogen to fibrin during thrombus formation, crosslinking of the latter by the action of factor XIIIa during thrombus stabilization (e.g., D-Dimers) as well as lysis of the fibrin molecules through the action of plasmin upon healing (e.g., euglobulin lysis time, thrombin time, reptilase time) are further recommended measurements. It should be noted that the current norm (ISO 10993-4) particularly recommends the measurements of TAT and F1+2 for thrombin formation and fibrinopeptide A for fibrin generation.

5. Conclusion

To facilitate interstudy and interlaboratory comparisons, a reproducible assessment and categorization of the in vitro thrombogenicity—as one biofunction of blood contacting materials—is mandatory and should ideally be approached as a joint scientific effort. Here, we reviewed the status of the in vitro characterization of blood–materials interactions in view of test parameters and methods as well as test systems and reference materials. Furthermore, prerequisites for a reproducible testing were described. As an initial step, a recent round-robin study showed that the platelet activation and adhesion on polymer-based implant materials with different chemical and physical material properties can be assessed reproducibly and with identical categorization in the participating labs. As a next step, multicentric studies with whole blood are needed so that—perspectively—parameters of all test categories can be assessed under harmonized and standardized conditions.

Beyond testing with blood from apparently healthy human subjects, some significant questions remain unanswered. Should blood from the device recipients be used for testing? As recently reported, values substantially differ from those of healthy subjects. Which animal species are appropriate for testing? Great species differences are known in respect to blood plasma proteins and platelet function. The short-term interactions were described to be comparable in vitro and in vivo, however, a prediction of the long-term behavior seems questionable.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomaterials, blood tests, implants, in vitro, thrombogenicity

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