Are there sufficient standards for the in vitro hemocompatibility testing of biomaterials?

Steffen Braune, Michael Grunze, Andreas Straub, and Friedrich Jung

Citation: Biointerphases **8**, 33 (2013); doi: 10.1186/1559-4106-8-33
View online: https://doi.org/10.1186/1559-4106-8-33
View Table of Contents: http://avs.scitation.org/toc/bip/8/1
Published by the American Vacuum Society

Biointerphases
a SpringerOpen Journal

PERSPECTIVE Open Access

Are there sufficient standards for the *in vitro* hemocompatibility testing of biomaterials?

Steffen Braune[1], Michael Grunze[2], Andreas Straub[3] and Friedrich Jung[1*]

In this perspective we address a fundamental and unresolved issue in biomaterials science: *What are the molecular mechanisms which make a material blood compatible - or not?* Despite the widespread use and the billion-dollar industry producing medical devices and implants, there is still a lack of fundamental understanding of the –admittedly– complicated and interlinked reactions that occur when an artificial material is exposed to blood. The increasing number of clinical reports about *in vivo* dysfunctional cardiovascular devices underlines the importance to understand –and be able to predict– the pathophysiological events occurring upon contact of blood with "hemocompatible" materials. The missing knowledge about the pathophysiological processes in the material/blood interphase and the lack of reliable correlations between *in vitro-in vivo* experiments, hinders us to develop scientific design principles to avoid complications in a clinical situation and achieve the optimal and lasting treatment. One may ask why this is the case, despite the huge research efforts taking place in laboratories worldwide to improve existing biomaterials and to develop new and better ones. One reason is certainly the analytical, intellectual and financial efforts it takes to unravel the interlinked biochemical reactions taking place in the interphase between the material and the body. But what actually slows the scientific community from developing models and theories on hemocompatibility is the fact that hardly any publication targeted at developing hemocompatible biomaterials conducts and describes the experiments and results in enough detail to allow a direct comparison to data in the literature or results of other laboratories. The lack of defined and certified positive or negative standards and experimental protocols even prevents us from concluding if a material is *relatively* better than another material, which may or may not be in use in medical practice. Hence, the literature is full with claims for "new" or "better" biomaterials, which have not undergone an *in vitro* side by side comparison to a standard, nor animal tests or clinical evaluation. If such *in vitro* comparisons with accepted standards would be performed according to standardized protocols, respective reports could contribute in a meaningful way to a database, which ultimately would provide the basis for a roadmap to better implants.

This perspective is written to remind the reader of the complicated and interlinked reactions taking place on a material surface upon contact with blood, which may lead to inflammation and thrombosis. It is not our intention to give a comprehensive review on hemocompatibility testing, but to emphasize the challenges in establishing international standards in this area of biomaterial research and to motivate the academic community to define the required standards for their own benefit and for this research area in general.

Background

The increasing age of populations in industrialized nations - together with a lack of physical activity and changed diet habits - are associated with an increase of atherosclerotic diseases causing high morbidity and mortality [1]. Atherosclerosis is caused by a complex interplay of endothelial dysfunction, lipid disturbances, platelet activation, thrombosis, oxidative stress, vascular smooth muscle activation, altered matrix metabolism, re-modelling, genetic factors, and inflammation [2]. These phenomena cause plaque formation in arterial walls, which leads to a progressive narrowing of the arterial lumen and an insufficient supply of the adjacent tissue with oxygen and nutrients. Atherosclerotic diseases still cannot be cured and, till today, about 50% of the population of industrial nations die from cardiovascular diseases. To restore sufficient blood flow in diseased arteries, balloon catheters with or without the implantation of endovascular stents [3,4] are routinely applied. These cardiovascular implants are selected and designed to be hemocompatible in order to fulfil their function. However, specific drawbacks limit their use, such as: long-term

* Correspondence: Friedrich.Jung@hzg.de
[1]Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstrasse 55, Teltow 14513, Germany
Full list of author information is available at the end of the article

endothelial dysfunction, delayed re-endothelialization, thrombogenicity and embolization, permanent physical irritation, inflammation at the implantation site, as well as non-permissive or disadvantageous characteristics for later surgical revascularization [5,6]. The resulting thrombotic occlusion can lead to total implant failure, as it is observed, for instance, as late in stent thrombosis for drug-eluting stents [7,8]. Similar undesired phenomena are reported for other cardiovascular implants and blood-contacting devices made of metals, polymers, and other artificial materials including vascular grafts, occluder systems, heart valves, ventricular assist devices, heart-lung machines, etc. [9-13].

In the complex and dynamic process of blood-material interaction, the composition of immediately adsorbed protein layers, but also conformational changes of surface adsorbed proteins are considered to substantially influence the cellular interactions (by the exposure of neo/cryptic epitopes) [14-18]. Key elements of the latter are blood platelets, which play a central role in physiological and pathological processes of hemostasis, inflammation, tumor-metastasis, wound healing, and host defence [19-24]. An understanding of the interactions of proteins, platelets and the surfaces of blood contacting biomaterials is, consequently, crucial if we aim to identify and design truly hemocompatible biomaterials.

Despite many years of research, the detailed mechanisms of pro-coagulatory and pro-inflammatory events upon blood contact with artificial surfaces are only poorly understood [25,26]. This lacking knowledge, the absence of common standards in the *in vitro* testing and the missing consensus of what makes a material blood incompatible *in vivo* has led to the term "blood compatibility catastrophe" (coined by Buddy Ratner) [27,28]. Although standards for hemocompatibility testing are defined in the ISO 10993–4, this catalogue comprises minimum test requirements only and supplementary tests need to be performed [29]. A standardized *in vitro* hemocompatibility test panel should enable the detection and elimination of undesired and excessive material-induced thrombosis and inflammatory events at an early stage of biomaterial development.

In the following, we discuss currently applied approaches and suggest practises and standards for *in vitro* hemocompatibility testing. If we want to understand the complex interplay and the underlying pathophysiological mechanisms, both players, the biomaterial surface and the blood cells/components, need to be thoroughly studied and their interactions require characterization in greatest possible detail. Moreover, knowledge about origin and composition and, ultimately, the genetic fingerprint of the donor may prospectively lead towards a more "personalized" medicine.

Characterization of the material surface

The analytical and surface science methods which can be used to quantitatively analyze the implant material surfaces *ex situ* are well described in the literature, in particular with respect to biomaterials characterization in the book edited by Ratner et al. [26]. Surface analysis can routinely specify the elemental and chemical composition of the surface with a few percent accuracy using X-ray photoelectron spectroscopy (XPS), secondary ion spectroscopy (SIMS) and, in the case of polymers, with vibrational spectroscopies such as infrared absorption spectroscopy (IR) and RAMAN spectroscopy. The identification of minority species on the surface which may cause inflammatory events, however, is more difficult to access but equally important. Also, the potential of impurity diffusion (e.g. solvent residues in polymers) from the bulk to the blood contacting surface should be carefully checked. It is therefore highly desirable to confirm also the bulk composition, and to determine any concentration gradients in composition (e.g. in alloys or polymer blends) from the bulk to the interface. Depth profiling is done by angle dependent measurements in XPS, but also by changing the photon energies or the detection mode (electrons or photons) when synchrotron radiation is used.

It is quite common that the bulk composition of alloys deviates considerably from batch to batch, and that polymers and polymer mixtures contain softeners and solvent residues. This means that an unexpected surface composition is not necessarily due to contaminations or segregation; it could be that the material is of different composition than expected. Hence, for commercial materials the composition information provided by the supplier should be checked for every batch of samples used.

It has also to be considered that measuring the surface composition of a material in vacuum (e.g. when using electron spectroscopies) or in an ambient atmosphere (e.g. vibrational spectroscopies) does not necessarily reflect the surface composition (and morphology) in an aqueous environment or blood. *In situ* measurements in aqueous solutions are much more complicated -if not impossible in some cases- and require specialized instruments (e.g. nonlinear optical techniques) not readily available in most laboratories.

However, it is not only the chemical make-up of the interface, which is important. Inflammatory and pro-coagulant processes can be induced by intrinsic physical biomaterial properties such as porosity, roughness, charge and charge density, elasticity, or surface energy (wettability) [30-32]. Therefore, a profound physical-chemical characterization of the material properties is mandatory and required for the interpretation of material-protein as well as material-cell interactions. Water wettability is a routinely determined and important parameter, but not an indicator if a surface is biocompatible or not. More relevant is the surface energy, measured by the contact angle of several liquids [33]. The measurements on the physical properties of

the materials surface should be done in a dry and a hydrated state, since contact with aqueous solutions will lead to swelling of most polymers and, hence, to a different morphology and stiffness, or in the case of polymer mixtures and block-copolymers to a different surface composition. All these parameters should be measured under standardized conditions after sterilization, and selectively confirmed before application of the implant. The latter is in particular true for materials that are prone to oxidation in air, which will lead to a change in surface composition. A hydrocarbon contamination layer adsorbed from the ambient can also influence the initial reaction with bodily fluids. A quick indicator for changes in surface composition is, for example, the water contact angle.

It is recommended to characterize representative samples for their general properties after preparation, and, importantly, prior to the actual experiments, after final cleaning steps and sterilization [34]. All mechanical, surface morphology, and roughness measurements should be done after preparation. Surface composition should be checked on samples that underwent final cleaning and sterilization since the latter can majorly influence the material properties [35].

The chemical composition of the bulk material and the surface layer has to be specified as precisely as possible. Surface composition conveniently is measured with the instruments available at the laboratory; however, an XPS analysis of the surface is required as an absolute measurement of composition and for cross calibration of other characterization methods. Mandatory is that the conditions under which the measurements were taken are meticulously specified (e.g. for XPS: X-ray source, power, photon energy, instrumental resolution, photon incidence angle, take of angle, vacuum conditions, statistics of the measurements with error margins, indications of radiation damage, …) and the methods and assumptions used to determine surface concentrations and depth distributions are described in sufficient detail. It has to be checked if the distribution of elements or functional groups is homogeneous, or patchy. Polymer surfaces should be characterized by IR and RAMAN spectra, including an assignment of the vibrational bands. If there are questions about the surface composition, a deconvolution of the spectra is needed for a consistent assignment of absorption bands.

Before exposure to the biological medium, a final check of the water contact angle, and comparison of the value to the contact angle measured on the pristine and cleaned material, is an ambiguous but useful indicator if the surface has been contaminated by impurities.

In an ideal setting, the materials under study will be tested side by side to an established and defined materials standard. Only a combination of cross-calibrated surface analysis techniques can possibly establish a correlation between surface characteristics and hemocompatibility. In order to have a final judgment which techniques are most useful and essential to make a correlation between surface properties and hemocompatibility, a comparative study under standardized conditions of different materials has to be done. The more information is available about the surface, the easier it will be to make a comparison to other data and identify possible small but subtle differences in composition, which are then amplified in the hemocompatibility tests.

Endotoxin testing

It is well known that polymer-based biomaterials can be contaminated e.g. with pyrogenic substances including endotoxins and other microbial products [36-38]. Such substances may lead to non-specific immune reactions that can be characterized by e.g. macrophage activation, the generation of reactive oxygen species (ROS), activation of the complement system and the secretion of inflammatory cytokines from leukocytes [39]. The cellular response of immune cells towards microbial products is mediated by engagement of toll-like receptors, which are expressed on leukocytes and platelets [40,41]. Particularly, the binding of endotoxins to TLR4, the receptor for endotoxins, can result in the secretion of pro-inflammatory mediators and activation of inflammatory cells.

It strictly needs to be proven that candidate implant materials are free of soluble as well as of material-adherent microbial products. Failing this, contaminations of the material may lead to an erroneous evaluation of the material's hemo- and/or tissue-compatibility. Free soluble endotoxins can be routinely assessed, e.g. by commercially available and well established Limulus Amebocyte Lysate (LAL) based tests systems [37,38]. In addition, material-bound endotoxins should be determined, for instance, utilizing cell-based assays that analyse the viability and activation (cytokine secretion) of macrophages (RAW-blue™ cells) subsequent to direct material contact [36,42]. The combination of these "indirect" and "direct" test systems may allow an adequate evaluation of the material endotoxins burden [43].

Cytocompatibility testing

Cytotoxicity, the ability of a material to influence cellular viability (e.g. cell membrane integrity and growth), should be assessed as a primary biocompatibility test. International standards for cytotoxicity testing are determined in the EN DIN ISO standards (especially 10993–5, 10993–12 and ISO 7405) but, depending on the scientific background of the study, approaches differ clearly between laboratories. Screening of new candidate biomaterials can be performed with material eluates to determine the release of potentially toxic soluble substances from the material bulk (indirect test). This approach

may be well suited for a standardized testing, particularly in view of high-throughput screenings [44]. Since such setups are performed independently from the intended material application, results may be of limited scientific value but provide the basis for further testing of the direct cell-material interactions (direct test). In this case, the selection of site-specific cells (e.g. endothelial cells and smooth muscle cells) may allow a more application relevant evaluation of the material performance (e.g. cardiovascular applications) [45,46]. If a material surface is intended to inhibit the interaction with proteins and cells by antifouling properties, the direct setup cannot provide a reasonable statement and the toxicity has to be evaluated utilizing the indirect setup [47]. To enable an internal and inter-institutional comparison, it is recommended to evaluate the cytotoxicity of materials according to the above mentioned EN DIN ISO standards. Two test procedures are suggested: test of material extracts on adherent cells, e.g. L929 mouse fibroblast (indirect test) and, in addition, tests with direct contact of material and cells (direct test) [34,48,49]. For an indirect testing, polymer samples are exposed to serum-free cell culture medium under continuous stirring at 37 °C for three days. The resulting extract replaces the cell culture medium for L929 cells, which are seeded on polystyrene-based cell culture plates and are allowed to grow a sub-confluent layer. After 48 hours of incubation, cell morphology (e.g. phase-contrast microscopy), the viability of the cells (e.g. fluorescein diacetate/propidium iodide assay), integrity of the cell membrane (e.g. lactate dehydrogenase assay) and mitochondrial activity (e.g. MTS tetrazolium assay) is analysed. For direct testing, material samples are inserted in multiwell plates and seeded with cells. After reaching 80% confluence, assays are performed according to the indirect setup. Obtained results can be transformed in an assay-based and final score, which allows the discrimination of non-toxic and cytotoxic materials. Tissue culture treated polystyrene surfaces (negative) and Triton® X-100 treated cells (positive) can be applied as controls, independently from other, more specific, assay internal reference materials [45,50].

Hemocompatibility testing

As required by regulatory agencies, the hemocompatibility of biomaterials for medical devices has to be evaluated conforming standards defined in the ISO 10993–4. Thrombosis, however, is still noted in many of the clinically applied devices. The approval by the currently recommended panel of tests, consequently, does not guarantee clinical device/biomaterial hemocompatibility, which emphasized the major need for improved and *in vivo* predictive *in vitro* test setups. Unsolved concerns are also the lacking standards for anticoagulation or reference materials. Thus, variations between studies hardly enable a classification of hemocompatible and non-hemocompatible materials [51].

Anticoagulation of blood samples is mandatory in order to avoid spontaneous coagulation processes *in vitro*. Functional aspects of various anticoagulants have been reviewed extensively and are therefore not part of this perspective [52-54]. Most commonly used anticoagulants are heparin for whole blood studies and sodium citrate for studies that focus on platelet-biomaterial interactions *in vitro* [52,55]. More recently, hirudin appears in an increasing number of hemocompatibility studies and clinically applied routine test systems [56,57]. The decision for a specific anticoagulant should be driven by the clinical background, the choice of tested blood (e.g. whole blood, platelet rich plasma (PRP) or platelet poor plasma (PPP)) and the specific scientific questions of the study (e.g. basic screening of various materials or comprehensive investigation of the inflammatory and coagulatory potential of one material). In addition, it is crucial to determine and standardize the appropriate concentrations of the respective anticoagulant to ensure the functionality of blood cells and blood components, but also to minimize the inhibition of coagulation activation in the *in vitro* test system. No internationally accepted guidelines are established that define appropriate concentrations for anticoagulants used in the *in vitro* evaluation of biomaterial's hemocompatibility. Therefore, a strong need exists to define these parameters to enable an inter-institutional comparison of different studies and materials.

Heparin is routinely applied as systemic anticoagulant in many therapeutic approaches and, therefore, applied by many laboratories to investigate the hemocompatibility of biomaterials in whole blood studies. However, no common standard is established for heparin and different functional forms e.g. unfractionated heparin (UFH), low molecular weight heparin (LMWH) or pentasaccharide types (e.g. Fondaparinux) [58,59] are applied in varying concentrations (0.5 IU to 5 IU heparin per mL blood) [60-64]. For PRP based assays, concentrations of 0.105 – 0.109 M and 0.129 M tri-sodium citrate are recommended by the British Committee for Standards in Haematology (BCSH) and International Society on Thrombosis and Haemostasis (ISTH) [65-67]. These assays focus on the platelet-biomaterial interactions and are well suited to assess the thrombogenic potential of a material, even though, sodium citrate is not applied as a systemic anticoagulant. For *in vitro* studies focusing on complement activation, anticoagulation with hirudin (commercially available as lepirudin or bivalirudine [68]) appears to mimic physiological conditions more appropriately than other anticoagulants [56,57,69].

Standardized hemocompatibility testing is usually performed with blood from healthy subjects, which should be stringently characterized, free of medication (particularly platelet function inhibitors), should be non-smokers, avoid

vigorous exercise as well as certain nutrition that is known to influence platelet function [52]. Material systems at an advanced stage of development should additionally be tested with blood from the target-patient group to study how the disease and the therapeutical medication influence the material properties and if the functionality of the device is modified [70].

Guidelines for blood collection and preparation are extensively described in the ISTH and BCSH-guidelines [65,66,71]. The latter recommend minimizing venostasis during blood withdrawal, the use of a needle of 21 gauge and the collection of blood into plastic (polypropylene) or siliconized glass tubes. Despite recent studies question the necessity of a discard tube, it is further noted that the first 3 – 4 mL of blood are not suitable for testing due to the potential coagulation activation by e.g. tissue factor (thromboplastin) in the syringe [72-74]. Subsequent to the blood withdrawal and prior to the final test, blood samples should rest for at least 30 minutes under mild agitation, since platelet function, particularly platelet aggregation can be reduced immediately after blood collection [75]. The total test duration should not exceed 4 hours to ensure an appropriate function of blood cells and blood plasma proteins [29,76-80].

Pre-analytical blood testing should be routinely carried out to verify the suitability of blood donors. Participants who exhibit latent inflammation, an altered platelet function or platelet disorders can, thus, be excluded [52]. Pre-tests should, at least, comprise haemogram-analysis, acute-phase protein determination as well as spontaneous and induced platelet function analysis.

Recommendations for a further preparation of whole blood comprise the sourcing of PRP and cell poor plasma and are summarized in the international guidelines for thrombosis and haemostasis testing [65,66,71,81,82]. These guidelines recommend that blood preparation should be carried out at room temperature and variations in temperature should be avoided due to the activation of blood cells at decreased temperatures [83,84]. However, there is evidence to suggest that a temperature of 37 °C, constantly kept during the blood withdrawal, storage and preparation as well as during the final assay, may reflect the *in vivo* situation even more appropriately [85-87].

Currently, various *in vitro* static and dynamic test systems are applied to evaluate the hemocompatibility of biomaterials. Tests under static conditions were earlier developed by Breddin to determine platelet spreading as a diagnostic and prognostic marker for platelet disorders, e.g. thrombocytopenia [88]. More recently, this method was adapted for hemocompatibility screening studies [89] and further modified and automated for thrombogenic materials [90-92]. Static test systems simulate low flow or even no flow areas in the vasculature and provide a rapid and sensitive setup to determine the thrombogenicity of a biomaterial, in particular platelet adhesion. Dynamic systems are more appropriate to simulate the conditions in flowing whole blood *in vivo* or of extracorporeal devices and allow an adaptation of the test conditions to the (patho-) physiological situation of specific medical devices [93,94]. Different setups are currently used including: agitators, centrifugation systems, flow chambers, chandler systems and closed loop circulation models. However, information about the test conditions and reproducibility are required but lacking for many of these systems. An appropriate dynamic model should simulate the arterial or venous conditions of blood/material interactions during the clinical application. Size and geometry of the device, duration of contact (up to 240 minutes), temperature as well as rheological (flow) and shear conditions should be considered in the design of the setup [29,52,70]. If a miniaturization of the device is required, blood flow should be proportionally adjusted to the reduced material surface area in order to adapt the degree of mechanically induced shear forces [70]. In studies focusing on platelet-material interactions, elevated shear forces ($> 50\ N \cdot m^{-2}$), as well as recalcification of citrated blood samples or air in the test system may artificially induce platelet activation/aggregation and should be avoided to ensure an accurate determination of thrombogenic effects induced explicitly by the material surfaces and not by the experimental conditions [95-97]. Especially in the dynamic test systems, kinetic measurements should preferentially be performed instead of endpoint determinations to characterize the time-dependence of blood/material interactions.

To classify a material hemocompatible, reference materials have to be defined, which enable the discrimination between known hemocompatible materials and non-hemocompatible materials. Respective reference materials were recommended in earlier national or international consensus initiatives [98-100]. Unfortunately, these materials are not frequently used, in part not commercially available and may also not represent current state-of-the-art technology. As a consequence, a variety of references aside of the recommended materials (filler free polydimethylsiloxan, low density polyethylene, polyvinylchloride, polyurethane, cellulose and expanded polytetrafluorethylen) have been applied in recent studies [51,100,101].

To overcome these problems, one promising approach may be to determine the reference materials in view of the clinically applied gold standards. This primarily allows an application specific comparison but potentially is also suitable for standardization in basic screening studies. In the context of positive controls, efforts were made to establish polyacrylate-based materials, which strongly interact with human platelets [102]. Alternatively, collagen coated glass or polystyrene, also served as an appropriate and standardized positive control. In order to allow an inter-study and inter-institutional

comparison, routine controls as used in clinical chemistry and hematology have to be performed for each test parameter [103-105]. In test setups that focus on the interaction of blood with eluates or micro- and nano-particles, the use of soluble pro-coagulatory substances e.g. thrombin or ADP may be appropriate [106,107].

The use of a score system, as suggested by Seyfert (single test scores are summarized in a total score to evaluate the hemocompatibility of the sample), might further help to standardize the *in vitro* hemocompatibility testing of biomaterials [29]. Alternatively, acceptance criteria can be applied with comparison to a predicate type material. The criteria can be expressed as percentage of baseline and should, at least, include hemolysis, thrombosis (platelet covered surface area), platelet retention/count as well as coagulation time [51].

As a consequence of the currently recommended but insufficient test panel defined in the ISO norm, further tests should be considered for assessing hemocompatibility of biomaterials.

In order to understand which specific plasma proteins are primarily adsorbed by respective surfaces, adsorption profiles of proteins can be recorded using ELISA and/or western blotting techniques [16,108-111]. Further comprehensive test collections reported by Seyfert et al. comprise tests for the following aspects: contact activation, fibrinogen-fibrin conversion, fibrinolysis, hemolysis, proteolysis, and platelet activation [29]. In addition to these tests, also the activation of the plasmatic coagulation (e.g. quantified by the measurement of thrombin-antithrombin complexes and/or prothrombin fragments 1 + 2 [112]) as well as the interaction of granulocytes, monocytes and lymphocytes with the biomaterial surface might be beneficial to understand the complex interplay between thrombotic and inflammatory processes [113]. In this context, also whole blood cytokine secretion and complement activation assays can be applied to clarify whether the thrombotic potential of a material is induced directly by the adhesion of platelets or by alternative inflammatory pathways [42,114]. As an example, complement factors (e.g. C5b-9) but also platelet agonists including thrombin and collagen support the shedding of procoagulant and proinflammatory microvesicles from the platelet surface, which show similar surface expression of activation dependent adhesion molecules (P-selectin, CD40L) as stimulated platelets [115-117].

Aside from the basic hemocompatibility screening, a fundamental understanding of the interactions between blood cells/components and body foreign surfaces is needed. Therefore, systematic studies have to be carried out, focusing the influence of distinct material properties on a molecular level. Knowledge about the material induced outside-in signal transduction may allow new material design approaches, which could provide improved hemocompatible surfaces. In the clinical situation, this could enable cessation or at least dose reductions of anti-platelet/anticoagulatory treatment regimens like for example after coronary artery stenting, where platelet inhibitors are administered or in ventricular assist devices (VAD) patients, where heparin, warfarin, phenprocoumon and platelet inhibitors are employed [118,119]. Thereby, life-threatening bleeding complications in these patient groups could be strongly reduced. In addition to an improvement of the properties of blood-contacting materials, the development of new antithrombotic therapies based on the blocking of undesired pathways with potentially less bleeding complications compared to current treatment strategies may also be conceivable [120].

Conclusion

This perspective pointed out some serious deficiencies in our understanding of "hemocompatibility" and summarized current approaches for *in vitro* hemocompatibility testing. While it is widely accepted that the standards given in the EN ISO 10993-4/5 are minimum requirements, it is still under debate which supplementary tests have to be performed for understanding the interaction between blood cells/components and artificial surfaces. To exclude activation of coagulatory and inflammatory processes induced by manufacturing processes of the candidate materials, standards for the preparation and characterization need to be defined that involve cleaning, sterilization, endotoxin determination as well as a profound physical-chemical characterization. Currently, there is no agreement which anticoagulant in which concentration should be used and what kind of pre-analytical test should be routinely performed to characterize and proof the functionality/suitability of the donated blood. Also, the choice of common reference materials for positive and negative controls has to be redefined, since materials suggested in earlier consensus papers may not represent state of the art technology or are not commercially available. Therefore, the main question of our manuscript- namely whether there are sufficient standards for hemocompatibility testing of biomaterials -needs to be answered with "no" at the current point of time. A consensus in the above mentioned matters is highly desirable and a prerequisite for an internal and inter-institutional comparison of hemocompatibility studies as performed in clinical chemistry and heamatology. Moreover, a common approach may help to improve the *in vivo* predictability of *in vitro* hemocompatibility studies and, hopefully, will lead to a better understanding and advanced design of artificial biomaterials.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed equally to the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The authors thank the Helmholtz Association for funding of this work through Helmholtz-Portfolio Topic "Technology and Medicine".

Author details
[1]Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstrasse 55, Teltow 14513, Germany. [2]Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT), Hermann-von-Helmholtz-Platz 1, Eggenstein-Leopoldshafen 67344, Germany. [3]Department of Anesthesiology and Intensive Care Medicine, University Hospital, Hoppe-Seyler Street 3, Tübingen 72076, Germany.

Received: 10 September 2013 Accepted: 6 November 2013
Published: 25 November 2013

References

1. Butler D (2011) UN targets top killers. Nature 477:260–261
2. Sanon S, Lenihan DJ, Mouhayar E (2011) Peripheral arterial ischemic events in cancer patients. Vasc Med 16:119–130
3. Lumsden AB, Davies MG, Peden EK (2009) Medical and endovascular management of critical limb ischemia. J Endovasc Ther 16:II31–II62
4. Aoki J, Lansky AJ, Mehran R, et al. (2009) Early stent thrombosis in patients with acute coronary syndromes treated with drug-eluting and bare metal stents: the acute catheterization and urgent intervention triage strategy trial. Circulation 119:687–698
5. Jung F, Wischke C, Lendlein A (2011) Degradable, multifunctional cardiovascular implants: challenges and hurdles. MRS Bull 35:607–613
6. Erne P, Schier M, Resink TJ (2006) The road to bioabsorbable stents: reaching clinical reality? Cardiovasc Intervent Radiol 29:11–16
7. Joner M, Finn AV, Farb A, et al. (2006) Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. J Am Coll Cardiol 48:193–202
8. Rossi ML, Zavalloni D, Gasparini GL, Presbitero P (2008) Very late multivessel thrombosis of bare metal stents with concomitant patent drug-eluting stents after withdrawal of aspirin. Int J Cardiol 131:e7–e9
9. Desai M, Seifalian AM, Hamilton G (2011) Role of prosthetic conduits in coronary artery bypass grafting. Eur J cardio-thoracic Surg 40:394–398
10. Park J-W, Leithäuser B, Gerk U, et al. (2009) Percutaneous left atrial appendage transcatheter occlusion (PLAATO) for stroke prevention in atrial fibrillation: 2-year outcomes. J Invasive Cardiol 21:446–450
11. Schoen FJ, Padera RF, Jr (2013) A - Substitute Heart Valves. In: Ratner BD, Hoffman A, Schoen FJ, Lemons JE (ed) Biomaterials Science - An Introduction to Materials in Medicine, 3rd edition. Elsevier Academic Press, Amsterdam, Heidelberg [a.o.], pp 761–771
12. Sims DB, Takayama H, Uriel N, et al. (2011) Ventricular assist device-associated thrombus. Circulation 124:e197–e198
13. Song Y, Feijen J, Grijpma DW, Poot AA (2011) Tissue engineering of small-diameter vascular grafts: a literature review. Clin Hemorheol Microcirc 49:357–374
14. Sivaraman B, Latour R (2010) The relationship between platelet adhesion on surfaces and the structure versus the amount of adsorbed fibrinogen. Biomaterials 31:832–839
15. Sivaraman B, Latour R (2010) The adherence of platelets to adsorbed albumin by receptor-mediated recognition of binding sites exposed by adsorption-induced unfolding. Biomaterials 31:1036–1044
16. Tengvall P (2011) Protein Interactions with Biomaterials, Compr. Biomater. Elsevier Ltd., Oxford, pp 63–73
17. Yu K, Lai BFL, Kizhakkedathu JN (2012) Carbohydrate structure dependent hemocompatibility of biomimetic functional polymer brushes on surfaces. Adv Healthc Mater 1:199–213
18. Cheng X, Canavan HE, Graham DJ, et al. (2006) Temperature dependent activity and structure of adsorbed proteins on plasma polymerized N-isopropyl acrylamide. Biointerphases 1:61
19. Jurk K, Kehrel BE (2005) Platelets: physiology and biochemistry. Semin Thromb Hemost 31:381–392
20. Gori T (2011) Viscosity, platelet activation, and hematocrit: progress in understanding their relationship with clinical and subclinical vascular disease. Clin Hemorheol Microcirc 49:37–42
21. Weyrich A, Cipollone F, Mezzetti A, Zimmerman G (2007) Platelets in atherothrombosis: new and evolving roles. Curr Pharm Des 13:1685–1691
22. Semple JW, Italiano JE, Freedman J (2011) Platelets and the immune continuum. Nat Rev Immunol 11:264–274
23. Reinhart WH (2013) Platelets in vascular disease. Clin Hemorheol Microcirc 53:71–79
24. Nurden AT (2011) Platelets, inflammation and tissue regeneration. Thromb Haemost 105(Suppl):S13–S33
25. Sefton MV (2012) Nonthrombogenic materials and strategies: case study. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE (ed) Biomaterials science: an introduction to materials in medicine, 3rd edition. Elsevier Academic Press [a.o.], Amsterdam, Heidelberg, pp 785–760
26. Ratner BD (2012) Biomaterials science: an introduction to materials in medicine, 3rd edition. Elsevier, Academic Pr, Amsterdam, Heidelberg [a.o.]
27. Ratner BD (1993) The blood compatibility catastrophe. J Biomed Mater Res 27:283–287
28. Ratner BD (2007) The catastrophe revisited: blood compatibility in the 21st century. Biomaterials 28:5144–5147
29. Seyfert U (2002) In vitro hemocompatibility testing of biomaterials according to the ISO 10993–4. Biomol Eng 19:91–96
30. Thevenot P, Hu W, Tang L (2008) Surface chemistry influences implant biocompatibility. Curr Top Med Chem 8:270–280
31. Anderson JM, Rodriguez A, Chang DT (2008) Foreign body reaction to biomaterials. Semin Immunol 20:86–100
32. Anderson JM (1988) Inflammatory response to implants. ASAIO Trans Am Soc Artif Intern Organs 34:101–107
33. Marmur A (2009) Solid-surface characterization by wetting. Annu Rev Mater Res 39:473–489
34. Hiebl B, Lützow K, Lange M, et al. (2010) Cytocompatibility testing of cell culture modules fabricated from specific candidate biomaterials using injection molding. J Biotechnol 148:76–82
35. Rickert D, Lendlein A, Schmidt AM, et al. (2003) In vitro cytotoxicity testing of AB-polymer networks based on oligo(epsilon-caprolactone) segments after different sterilization techniques. J Biomed Mater Res B Appl Biomater 67:722–731
36. Roch T, Pierce BF, Zaupa A, et al. (2011) Reducing the endotoxin burden of desaminotyrosine- and desaminotyrosyl tyrosine-functionalized gelatin. Macromol Symp 309–310:182–189
37. Lieder R, Petersen PH, Sigurjónsson OE (2013) Endotoxins - the invisible companion in biomaterials research. Tissue Eng Part B Rev 19:391–402
38. Gorbet MB, Sefton MV (2005) Endotoxin: the uninvited guest. Biomaterials 26:6811–6817
39. Franz S, Rammelt S, Scharnweber D, Simon JC (2011) Immune responses to implants - a review of the implications for the design of immunomodulatory biomaterials. Biomaterials 32:6692–6709
40. Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. Cell 140:805–820
41. Rondina MT, Weyrich AS, Zimmerman GA (2013) Platelets as cellular effectors of inflammation in vascular diseases. Circ Res 112:1506–1519
42. Roch T, Cui J, Kratz K, et al. (2012) Immuno-compatibility of soft hydrophobic poly (n-butyl acrylate) networks with elastic moduli for regeneration of functional tissues. Clin Hemorheol Microcirc 50:131–142
43. Jones CF, Grainger DW (2009) In vitro assessments of nanomaterial toxicity. Adv Drug Deliv Rev 61:438–456
44. Yliperttula M, Chung BG, Navaladi A, et al. (2008) High-throughput screening of cell responses to biomaterials. Eur J Pharm Sci 35:151–160
45. Krüger A, Braune S, Kratz K, et al. (2012) The influence of poly(n-butyl acrylate) networks on viability and function of smooth muscle cells and vascular fibroblasts. Clin Hemorheol Microcirc 52:283–294
46. Wischke C, Krüger A, Roch T, et al. (2013) Endothelial cell response to (co) polymer nanoparticles depending on the inflammatory environment and comonomer ratio. Eur J Pharm Biopharm 84:288–296
47. Li S, Henry JJD (2011) Nonthrombogenic approaches to cardiovascular bioengineering. Annu Rev Biomed Eng 13:451–475
48. Scharnagl N, Hiebl B, Trescher K, et al. (2012) Behaviour of fibroblasts on water born acrylonitrile-based copolymers containing different cationic and anionic moieties. Clin Hemorheol Microcirc 52:295–311
49. Cooperstein MA, Canavan HE (2013) Assessment of cytotoxicity of (N-isopropyl acrylamide) and poly(N-isopropyl acrylamide)-coated surfaces. Biointerphases 8:19
50. Trescher K, Roch T, Cui J, et al. (2013) Test system for evaluating the influence of polymer properties on primary human keratinocytes and fibroblasts in mono- and coculture. J Biotechnol 166:58–64

51. Van Oeveren W (2013) Obstacles in haemocompatibility testing. Scientifica (Cairo) 2013:1–14
52. Jung F, Braune S, Lendlein A (2013) Haemocompatibility testing of biomaterials using human platelets. Clin Hemorheol Microcirc 53:97–115
53. Oudemans-van Straaten HM, Kellum JA, Bellomo R (2011) Clinical review: anticoagulation for continuous renal replacement therapy - heparin or citrate? Crit Care 15:202
54. Mousa SA (2010) Anticoagulants, antiplatelets, and thrombolytics. Methods Mol Biol 663. doi: 10.1007/978-1-60761-803-4
55. Ratner BD, Hoffman AS, Schoen FJ, Lemons JE (2004) Biomaterials Science - An Introduction to Materials in Medicine, 2nd edition. Elsevier Academic Press, Amsterdam, Heidelberg [a.o.], p 913
56. Hamad OA, Nilsson PH, Wouters D, et al. (2010) Complement component C3 binds to activated normal platelets without preceding proteolytic activation and promotes binding to complement receptor 1. J Immunol 184:2686–92
57. Bexborn F, Engberg AE, Sandholm K, et al. (2009) Hirudin versus heparin for use in whole blood in vitro biocompatibility models. J Biomed Mater Res A 89:951–9
58. Alquwaizani M, Buckley L, Adams C, Fanikos J (2013) Anticoagulants: a review of the pharmacology, dosing, and complications. Curr Emerg Hosp Med Rep 1:83–97
59. Bussey H, Francis JL, Consensus Group TH (2004) Heparin overview and issues. Pharmacotherapy 24:103S–107S
60. Chang X, Gorbet M (2013) The effect of shear on in vitro platelet and leukocyte material-induced activation. J Biomater Appl 28:407–15
61. Sperling C, Maitz MF, Talkenberger S, et al. (2007) In vitro blood reactivity to hydroxylated and non-hydroxylated polymer surfaces. Biomaterials 28:3617–25
62. Wendel HP, Hauser N, Briquet F, Ziemer G (2002) Hemocompatibility of medical connectors with biopassive or bioactive surface coatings. J Biomater Appl 17:5–17
63. Waterhouse A, Yin Y, Wise SG, et al. (2010) The immobilization of recombinant human tropoelastin on metals using a plasma-activated coating to improve the biocompatibility of coronary stents. Biomaterials 31:8332–40
64. Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD (2007) The role of complement in biomaterial-induced inflammation. Mol Immunol 44:82–94
65. Mackie I, Cooper P, Lawrie A, et al. (2013) Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol 35:1–13
66. Cattaneo M, Cerletti C, Harrison P, et al. (2013) Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost 11:1183–1189
67. Cattaneo M, Hayward CPM, Moffat KA, et al. (2009) Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. J Thromb Haemost 7:1029
68. Koster A, Fischer K-G, Harder S, Mertzlufft F (2007) The direct thrombin inhibitor argatroban: a review of its use in patients with and without HIT. Biol Targets Ther 1:105–12
69. Kopp R, Bernsberg R, Kashefi A, et al. (2005) Effect of hirudin versus heparin on hemocompatibility of blood contacting biomaterials: an in vitro study. Int J Artif Organs 28:1272–7
70. Vienken J (2007) The need for a systems approach in biomaterials testing. Med Device Technol 18(12–4):16–7
71. Lippi G, Salvagno GL, Montagnana M, et al. (2012) Quality standards for sample collection in coagulation testing. Semin Thromb Hemost 38:565–75
72. Smock KJ, Crist RA, Hansen SJ, et al. (2010) Discard tubes are not necessary when drawing samples for specialized coagulation testing. Blood Coagul Fibrinolysis an Int J Haemost Thromb 21:279–282
73. Raijmakers MTM, Menting CHF, Vader HL, Van Der Graaf F (2010) Collection of blood specimens by venipuncture for plasma-based coagulation assays: necessity of a discard tube. Am J Clin Pathol 133:331–335
74. Cole M, Price L, Parry A, et al. (2007) A study to determine the minimum volume of blood necessary to be discarded from a central venous catheter before a valid sample is obtained in children with cancer. Pediatr Blood Cancer 48:687–695
75. Kalb ML, Potura L, Scharbert G, Kozek-Langenecker SA (2009) The effect of ex vivo anticoagulants on whole blood platelet aggregation. Platelets 20:7–11
76. Adcock DM, Hoefner DM, Kottke-Marchant K, et al. (2008) Collection, transport, and processing of blood specimens for testing plasma-based coagulation assays and molecular hemostasis assays: approved guideline, CLSI document H21-A5 (ISBN 1-56238-657-3), 5th edition. , Wayne, Pennsylvania, p 6
77. Adcock D, Kressin D, Marlar RA (1998) The effect of time and temperature variables on routine coagulation tests. Blood Coagul Fibrinolysis 9:463–470
78. Rao LV, Okorodudu AO, Petersen JR, Elghetany MT (2000) Stability of prothrombin time and activated partial thromboplastin time tests under different storage conditions. Clin Chim Acta 300:13–21
79. Breddin HK (2005) Can platelet aggregometry be standardized? Platelets 16:151–8
80. Cohen MG, Rossi JS, Garbarino J, et al. (2011) Insights into the inhibition of platelet activation by omega-3 polyunsaturated fatty acids: beyond aspirin and clopidogrel. Thromb Res 128:335–40
81. Endler G, Slavka G, Perkmann T, Haushofer A (2010) The importance of preanalytics for the coagulation laboratory. Haemostaseologie 30:63–70. quiz 71–2
82. Favaloro EJ, Adcock Funk DM, Lippi G (2012) Pre-analytical variables in coagulation testing associated with diagnostic errors in hemostasis. Lab Med 43:1–10
83. Böhm M, Täschner S, Kretzschmar E, et al. (2006) Cold storage of citrated whole blood induces drastic time-dependent losses in factor VIII and von willebrand factor: potential for misdiagnosis of haemophilia and von willebrand disease. Blood Coagul Fibrinolysis an Int J Haemost Thromb 17:39–45
84. Favaloro EJ, Soltani S, McDonald J (2004) Potential laboratory misdiagnosis of hemophilia and von willebrand disorder owing to cold activation of blood samples for testing. Am J Clin Pathol 122:686–92
85. Polderman KH (2012) Hypothermia and coagulation. Crit Care 16:A20
86. Straub A, Breuer M, Wendel HP, et al. (2007) Critical temperature ranges of hypothermia-induced platelet activation: possible implications for cooling patients in cardiac surgery. Thromb Haemost 97:608–616
87. Straub A, Krajewski S, Hohmann JD, et al. (2011) Evidence of platelet activation at medically used hypothermia and mechanistic data indicating ADP as a key mediator and therapeutic target. Arterioscler Thromb Vasc Biol 31:1607–16
88. Breddin K (1968) In-vitro-Methoden zur Beurteilung der Plättchenfunktion. Blut 18:84–89
89. Frank RD, Dresbach H, Thelen H, Sieberth HG (2000) Glutardialdehyde induced fluorescence technique (GIFT): a new method for the imaging of platelet adhesion on biomaterials. J Biomed Mater Res 52:374–81
90. Braune S, Hönow A, Mrowietz C, et al. (2011) Hemocompatibility of soft hydrophobic poly(n-butyl acrylate) networks with elastic moduli adapted to the elasticity of human arteries. Clin Hemorheol Microcirc 49:375–90
91. Braune S, Alagöz G, Seifert B, et al. (2012) Automated image-based analysis of adherent thrombocytes on polymer surfaces. Clin Hemorheol Microcirc 52:349–55
92. Lange M, Braune S, Luetzow K, et al. (2012) Surface functionalization of poly (ether imide) membranes with linear, methylated oligoglycerols for reducing thrombogenicity. Macromol Rapid Commun 33:1487–92
93. Goldsmith HL, Bell DN, Braovac S, et al. (1995) Physical and chemical effects of red cells in the shear-induced aggregation of human platelets. Biophys J 69:1584–95
94. Goldsmith HL, Yu SS, Marlow J (1975) Fluid mechanical stress and the platelet. Thromb Diath Haemorrh 34:32–41
95. Schmid-Schonbein H, Rieger H, Fischer T (1980) Blood fluidity as a consequence of red cell fluidity: flow properties of blood and flow behavior of blood in vascular diseases. Angiology 31:301–319
96. Phillips DR, Teng W, Arfsten A, et al. (1997) Effect of Ca2+ on GP IIb-IIIa interactions with integrilin: enhanced GP IIb-IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. Circulation 96:1488–1494
97. Kalman PG, McCullough DA, Ward CA (1990) Evacuation of microscopic air bubbles from dacron reduces complement activation and platelet aggregation. J Vasc Surg 11:591–8
98. Lemm W (1992) The Reference Materials of the European Communities: Results of Hemocompatibility Tests. Springer, Berlin, Heidelberg
99. The European Society for Biomaterials (1991) 9th european conference on biomaterials chester, UK 9-11th september, 1991 in conjunction with 2nd consensus conference on definitions in biomaterials 7-8th september, 1991

and IUPAC working party on blood compatibility. J Mater Sci Mater Med 2:62–62
100. Brash JL, Ten Hove P (1993) Protein adsorption studies on "standard" polymeric materials. J Biomater Sci Polym Ed 4:591–599
101. Bélanger MC, Marois Y (2001) Hemocompatibility, biocompatibility, inflammatory and in vivo studies of primary reference materials low-density polyethylene and polydimethylsiloxane: a review. J Biomed Mater Res 58:467–77
102. Hansen A, McMillan L, Morrison A, et al. (2011) Polymers for the rapid and effective activation and aggregation of platelets. Biomaterials 32:7034–41
103. Kottke-Marchant K, Bruce D (2012) Laboratory Hematology Practice. Wiley-Blackwell, Chichester, Hoboken, p 776
104. Hoffman R, Jr EJB, Silberstein LE, et al. (2012) Hematology: Basic Principles and Practice, 6th edition. Churchill Livingstone Elsevier, Philadelphia
105. Kitchens CS, Konkle BA, Kessler CM (2013) Consultative Hemostasis and Thrombosis, 3rd edition. Saunders Elsevier, Philadelphia
106. Myerson J, He L, Lanza G, et al. (2011) Thrombin-inhibiting perfluorocarbon nanoparticles provide a novel strategy for the treatment and magnetic resonance imaging of acute thrombosis. J Thromb Haemost 9:1292–300
107. Koziara JM, Oh JJ, Akers WS, et al. (2005) Blood compatibility of cetyl alcohol/polysorbate-based nanoparticles. Pharm Res 22:1821–8
108. Brash JL, Uniyal S (1979) Dependence of albumin-fibrinogen simple and competitive adsorption on surface properties of biomaterials. J Polym Sci Polym Symp 66:377–389
109. Brash JL (2000) Exploiting the current paradigm of blood-material interactions for the rational design of blood-compatible materials. J Biomater Sci Polym Ed 11:1135–46
110. Cornelius RM, Shankar SP, Brash JL, Babensee JE (2011) Immunoblot analysis of proteins associated with self-assembled monolayer surfaces of defined chemistries. J Biomed Mater Res A 98:7–18
111. Rabe M, Verdes D, Seeger S (2011) Understanding protein adsorption phenomena at solid surfaces. Adv Colloid Interface Sci 162:87–106
112. Weber N, Wendel HP, Ziemer G (2002) Hemocompatibility of heparin-coated surfaces and the role of selective plasma protein adsorption. Biomaterials 23:429–39
113. Gorbet MB, Sefton MV (2004) Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials 25:5681–703
114. Levy M, Hartman AR (1996) Heparin-coated bypass circuits in cardiopulmonary bypass: improved biocompatibility or not. Int J Cardiol 53(Suppl):S81–7
115. Wiedmer T, Shattil SJ, Cunningham M, Sims PJ (1990) Role of calcium and calpain in complement-induced vesiculation of the platelet plasma membrane and in the exposure of the platelet factor Va receptor. Biochemistry 29:623–32
116. Neubauer H, Setiadi P, Pinto A, et al. (2009) Upregulation of platelet CD40, CD40 ligand (CD40L) and P-selectin expression in cigarette smokers: a flow cytometry study. Blood Coagul Fibrinolysis 20:694–8
117. Gemmell CH, Sefton MV, Yeo EL (1993) Platelet-derived microparticle formation involves glycoprotein IIb-IIIa, inhibition by RGDS and a Glanzmann's thrombasthenia defect. J Biol Chem 268:14586–9
118. Galper BZ, Mauri L (2013) Antiplatelet therapy after coronary stenting. Curr Treat Options Cardiovasc Med 15:1–10
119. Görlinger K, Bergmann L, Dirkmann D (2012) Coagulation management in patients undergoing mechanical circulatory support. Best Pract Res Clin Anaesthesiol 26:179–98
120. Cho MJ, Liu J, Pestina TI, et al. (2003) The roles of alpha IIb beta 3-mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. Blood 101:2646–51

doi:10.1186/1559-4106-8-33
Cite this article as: Braune *et al.*: Are there sufficient standards for the *in vitro* hemocompatibility testing of biomaterials?. *Biointerphases* 2013 **8**:33.