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Stirred, shaken, or stagnant: what goes on at the blood-biomaterial interface.

Ilya Reviakine,[1,2,*] Friedrich Jung,[3] Steffen Braune,[3] John L. Brash,[4] Robert Latour,[5] Maud Gorbet,[6] Wim van Oeveren.[7]

[1] Institute of Functional Interfaces (IFG), Karlsruhe Institute of Technology (KIT), Hermann von Helmholtz Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

[2] Department of Bioengineering, University of Washington - Seattle, WA, 98105, USA

[3] Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), Helmholtz Zentrum Geesthacht, Kantstrasse 55, 14513 Teltow, Germany

[4] Department of Chemical Engineering, School of Biomedical Engineering, McMaster University, Hamilton, Ontario, L8S 4L8, Canada

[5] Rhodes Engineering Research Center, Department of Bioengineering, Clemson University, Clemson, SC, 29634, USA

[6] Department of Systems Design Engineering, Biomedical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, N2L 3G1, Canada

[7] HaemoScan, Stavangerweg 23-23, 9723JC Groningen, the Netherlands

*Corresponding author: Ilya.Reviakine@kit.edu
Institute of Functional Interfaces (IFG)
Karlsruhe Institute of Technology (KIT)
Hermann-von-Helmholtz-Platz, 1
76344 Eggenstein-Leopoldshafen, Germany
Phone: +49 721 608 22991 / Fax: +49 721 608 23478

Stirred, shaken, or stagnant: what goes on at the blood-biomaterial interface.

Abstract

There is a widely recognized need to improve the performance of vascular implants and external medical devices that come into contact with blood by reducing adverse reactions they cause, such as thrombosis and inflammation. These reactions lead to major adverse cardiovascular events such as heart attacks and strokes. Currently, they are managed therapeutically. This need remains unmet by the biomaterials research community. Recognized stagnation of the blood-biomaterial interface research translates into waning interest from clinicians, funding agencies, and practitioners of adjacent fields. The purpose of this contribution is to stir things up. It follows the 2014 BloodSurf meeting (74th International IUVSTA Workshop on Blood-Biomaterial Interactions), offers reflections on the situation in the field and a three-pronged strategy integrating different perspectives on the biological mechanisms underlying blood-biomaterial interactions. The success of this strategy depends on reengaging clinicians and on the renewed cooperation of the funding agencies to support long-term efforts.

Keywords

Hemocompatibility; Cardiovascular implants; Biomaterials; Platelets; Coagulation; Antiplatelet therapy.

Research Agenda

Three strategies are proposed to address the recognized need for minimizing adverse reactions caused by the materials used in vascular implants:

- Testing to evaluate the performance of currently used materials. A particular emphasis should be placed on comparing different materials and results between different laboratories.
- Testing approaches should include surface-phase and fluid-phase reactions (thrombotic and embolic propensity) and encompass coagulation, platelet-, leukocyte-, and complement activation pathways.
- Modeling blood-biomaterial interface interactions beyond the limits of "physiologically relevant" conditions to uncover hidden degrees of freedom and cryptic interactions between components of various regulatory pathways.
- "Hacking" these pathways with the idea of incorporating the implant interface into their network.

Practice Points

- Mechanisms underlying the phenomena occurring at the blood-material interface are poorly understood. The questions facing the field of hemocompatibility have remained unchanged over the past several decades.
- Current clinical success of implants and other devices is brought about by the pharmacological strategies for managing the adverse reactions they cause.
- There are limits to the ability of pharmacological approaches to balance various risks associated with the specific pathologies, co-morbidities, and material-induced effects. This translates into dangers for the patient and costs for the healthcare systems. Hence, there is a pressing need for reducing the adverse effects of biomaterials.
- It is suggested that further progress in the hemocompatibility field will come from focusing on the details of the biological mechanisms underlying blood-biomaterial interactions.

1. Introduction: cardiovascular implants

The practice of modern medicine can hardly be envisaged without reliance on artificial materials in devices that substitute, or augment, the function of failing tissues and organs. In the context of blood-biomaterial interactions, examples of such devices include stents, mechanical heart valves, occluder systems, ventricular assist devices (VADs), synthetic vascular grafts, catheters, guidewires, as well as membranes of dialyzers, oxygenators, artificial heart-lung machines, and so on. Close to half a million cardiac stents are implanted in the US annually;[1] nearly three quarters of a million in Europe;[2] and around 100,000 in the UK.[3] Although stent overuse continues to be discussed,[4] it is clear that stenting is saving lives and improving the quality of life of millions of patients world-wide. Numbers for other implants are equally significant. In 2009, 90,000 heart valves were implanted in the US and 280,000 – world-wide.[5] The use of LVADs has increased considerably (from 246 patients in 2007 to ~ 2500 patients in 2014, according to the Interagency Registry for Mechanically Assisted Circulatory Support, or INTERMACS[6]), as mortality and complication rates decreased due to the design improvements; this success has generated some excitement in the clinical community.[7-10] Millions of patients undergo procedures requiring cardiopulmonary bypass (CPB), while the heart is stopped,[11, 12] thousands benefit from other forms of extracorporeal circulation, such as extracorporeal membrane oxygenation (ECMO), that is used particularly often in neonates.[13] Close to half a million patients are receiving hemodialysis treatment for end-stage renal disease in the US.[14] These devices involve contact between blood and artificial materials.

While native endothelium has naturally anticoagulant properties, the materials used in the artificial devices—metals and their oxides, polymers, pyrolytic and diamond-like carbon—activate the body's natural defense systems: coagulation, inflammation, and complement.[15, 16] Clinicians have become progressively better at managing the more severe of the resulting major adverse effects therapeutically through antiplatelet therapy (APT), anticoagulation therapy (ACT), or a combination of the two.

ACT based on intravenous (systemic) heparin and the introduction of heparin while priming the circuit is used to manage patients on CPB ever since CPB was developed in the 1950s,[11, 12, 17] ECMO patients,[18] and hemodialysis patients.[19] Of course, heparin is also the drug of choice for a wide variety of interventions, not only in the context of cardiovascular disorders. During percutaneous coronary intervention and stenting, it may be used together with antiplatelet agents such as abciximab, a GPIIb/IIIa antagonist.[20] For managing mechanical heart valves and VADs, long-term ACT is based on agents such as warfarin, by itself or in combination with APT.[21-24] VAD patient management also relies on heparin.[25]

Heparin causes serious complications, such as immune-mediated heparin-induced thrombocytopenia, in some patients.[26] Alternatives are therefore needed, especially in long-term applications such as ECMO and hemodialysis. These are reviewed by Fisher et al.[19] and include other thrombin inhibitors as well as regional citrate anticoagulation.[27] Most recent developments in this area are based on the recent understanding of the role contact activation plays in thrombosis vs. hemostasis and include factor XI and XII inhibitors that are being introduced into the clinic.[28, 29]

The breakthrough widely recognized as being transformative in catalyzing the wide-spread use of stents was the introduction in the 1990s of the aspirin-ticlopidine dual APT regimen (DAPT; ticlopidine was subsequently replaced by clopidogrel).[30-35] DAPT considerably reduced the thromboembolic complications associated with stent placement. It was also superior to the combination of anticoagulation therapy (ACT, consisting of intravenous heparin or phenprocoumon) with aspirin.[32] Further developments in the area of APT include the introduction of new P2Y12 and protease-activated receptor-1 antagonists into the clinical practice,[36-38] although the former appear to suffer from increased bleeding risks.[38] Finally, most recently, a dual-function agent (FXI inhibitor and GPIIb/IIIa antagonist) is being explored in animal studies for reducing stent thrombosis.[39]

Differences in the pharmacological strategies used to manage material-induced thrombosis induced by different devices are noteworthy. It is tempting to conclude that they reflect distinct

activation mechanisms at play in these situations, but it is also possible that they reflect the scale of the adverse reactions: smallest in the case of the stents, greatest in the case of the CPB, ECMO, and hemodialysis.

As far as implants and devices themselves are concerned, the history of CPB development is discussed in detail in several reviews.[11, 12, 17, 40] The key problem was the design of the oxygenator; first the use of animal and human (live donor) lungs was attempted, then bubble and rotating disk oxygenators were designed, followed by steel plate design, and finally, membrane and hollow fiber oxygenators were developed, which are used today. Moving away from the blood/gas interface of the bubble and rotating disk oxygenators, and reducing the surface area, improved their performance in terms of thrombosis; so did the introduction of heparin-coated circuits reviewed in refs. 41 and 42. These are used in ECMO, CPB, and hemodialysis devices to date, but their use not abolished the need for systemic heparin.

The history of mechanical heart valve design has been reviewed by Gott et al.[43] and Pibarot et al.[5] Several different types of mechanical heart valve prosthetics have evolved since the original caged ball design, with some becoming obsolete. The most recent advance in this area is represented by aortic valves designed for minimally-invasive, trans-catheter replacement.[44, 45] Similarly, VAD design went through two generations of improvements (with pulsatile and continuous flows).[22, 46] The design of these implants benefited from advances in material science and other adjacent engineering fields: for example, replacing titanium with pyrolytic carbon in heart valves reduced failure rates,[43] and fluid dynamics calculations that were combined with the understanding of platelet activation on non-physiological flow patterns improved the overall design.[5, 22, 47-49] The performance of these devices, however, continues to be limited by thrombotic complications and bleeding events associated with their pharmacological management.

The history of stents begins with the introduction of the steel wire-mesh stent (bare metal stent, BMS) by Ulrich Sigwart in 1986.[50] Since then, stent design and materials underwent one major change: Drug-eluting stents (DES), where the metal framework is coated with a polymer

matrix eluting anti-proliferative, anti-inflammatory, and immunosuppressive drugs, were developed to combat restenosis—the major complication of BMS. DES implantation in humans was first reported by several authors in 2001.[51-54] Their success in significantly reducing restenosis rates,[35, 55] and therefore the need for repeated interventions, translated into wide acceptance: DES are recommended by the latest European guidelines,[56] with over 75 % of stent implantations currently using DES.[1-3] However, studies have revealed that they suffer from late-stage thrombosis[57] noted as late as 5 years post-implantation,[58] necessitating the extension of the DAPT regimen (1 – 3 month for BMS, 6 – 12 month for DES[59]). This is also underscored in the latest European guidelines.[56] To quote Helft,[60] this "became a real nightmare for cardiologists". Here, the crucial role of the antiplatelet therapy (APT) is further substantiated by the risks associated with discontinuing the DAPT too early. The optimal duration of DAPT is still very much a subject of debate,[56, 60-62] despite improvements associated with the second-generation DES[63] (see Mukherjee et al. for the list of first- and second-generation stents[64]). Montalescot et al. review the results of seven different trials illustrating non-inferiority of short-term (3 – 6 month) as compared to long-term (1 year) DAPT after DES implantation, but comment on the limitations of these studies, such as the short follow-up times limiting their ability to detect late stent thrombosis.[65] These authors also reviewed studies where DAPT duration was longer than one year. Their overall conclusion was that "safe interruption of DAPT after 6 month may be possible in selected patients".[65] In one such long-duration DAPT study, Marui et al.[66] reported a significant reduction of the risk of stent thrombosis and major adverse cardiac events (MACE) associated with the extension of DAPT for an additional 18 month beyond 1 year after implantation of a first- or a second-generation DES. However, this carried with itself a higher risk of bleeding. Surprisingly, there was also an elevated risk of stent thrombosis and myocardial infarction during 3 month after discontinuation of DAPT independently of its duration.[66]

The mechanisms underlying late stent thrombosis in DES have been discussed in detail by Luscher et al.[57] and by Finn et al.[67] Essentially, they involve a delayed healing response that is thought to be caused by factors such as polymer toxicity, drug interactions, and strut geometry

that translate into excessive fibrin deposition on the strut surface, inflammation, and necrosis, contributing to the malapposition of the stent.[58, 63, 67]

Attempts to improve on the current state of affairs are proceeding in several directions. On one hand, there are the improvements to the DES design (thinner struts, different polymer coatings, and/or different drugs). Several authors reviewed the available clinical evidence for these second-generation DES stents pointing to improvements in safety while maintaining or improving their efficacy in terms of preventing restenosis.[55, 64, 65, 68] Nevertheless, existing guidelines stipulating the need to extended DAPT apply to both the first- and the second-generation DES, [56] and both were included in the trials referred to above,[65, 66] reflecting the remaining limitations.[69]

On the other hand, the strategy of promoting wound healing and stent integration through endothelialisation is represented by the endothelial progenitor cell (EPC) capture stents.[70] The first clinical investigation of these stents was reported by Aoki in 2005.[71] The results, however, were somewhat unconvincing,[72] leading to a combined EPC + DES strategy (in the form of the COMBO stent). The latest 1–year clinical data for this approach have been recently published.[73] The jury is still out on whether its performance will be superior to that of the DES stents, as discussed in a recent editorial by Mehran and Giustino.[69] So far, the recommended DAPT duration or the COMBO stent is 6 – 12 month.[73]

The third strategy being explored is that of bioresorbable stents. The idea here is to remove the foreign material from circulation over a period of time. It is represented by DES with bioresorbable coatings, or stents that are entirely made of biodegradable materials. In a 2015 report of the task force on the evaluation of coronary stents in Europe, "…bioresorbable coronary stents were not represented … due to absence of published evidence meeting the inclusion criteria at the time of the review."[55] In other words, the clinical evidence available so far is severely limited. We refer the readers to the most recent review by Lindholm and James discussing the technology.[74]

It is noteworthy that passive (barrier) coatings were not found to be useful on their own,[35] neither in stents, nor in other devices, although there is a stent design combining such a coating (based on phosphorylcholine) with drug elution (the Endeavor stent from Medtronics).[68] The subject of passive barrier coatings is discussed in more detail below in the context of coatings that resist the non-specific adsorption of proteins. This failure could be taken to highlight the importance of specific, biological aspects of the blood-biomaterial interactions over the generic physicochemical ones.

In summary, significant advances in ACT and APT have been made by clinicians, enabling pharmacological management of thrombotic complications arising from artificial materials in vascular implants and blood-contacting devices. Advances in the design of the devices have also been made, but by and large they were restricted to the mechanical aspects of their performance, *because the mechanisms underlying the phenomena occurring at the blood-material interface remain poorly understood*. In a nutshell, the factors, which need to be measured to evaluate material performance in blood, remain largely undefined. This underlies what Ratner had termed *the blood compatibility catastrophe*: the failure to deliver blood-compatible materials and predictive *in vitro* hemocompatibility tests.[15, 75]

2. State-of-the-art in the area of blood-biomaterial interactions and testing

That research into blood-biomaterial interactions has failed to produce an adequate material or an accepted *in vitro* test for evaluating the performance of materials in blood is hardly news: several articles have been dedicated to the subject. Nothing illustrates the stagnation in this field better than a series of quotes from papers spanning the last sixty years. In 1987, Spaet,[76] looking back at a ten-year period of blood compatibility meetings on one hand, and to the future on the other, asked "*What does a surface have to be to be 'physiological', and can this actually be synthesized by other than the appropriate cell in vivo?*" This question can be

traced back to the late 1800s / early 1900s, when (hydrophobic) vaseline and paraffin coatings on glass were shown to extend blood coagulation times.[77, 78] It is also echoed in the two articles by Ratner[15, 75] on the blood compatibility catastrophe published in 1993 and 2007. In 2016, we still don't have an answer.

In the same 1987 article, another question is raised: "*A topic that has never been suitably addressed is the relationship between in vitro and animal studies and the application of these studies to human disease*." This echoes a comment by Hastings (a onetime Chief of the Artificial Heart Program at NIH): "*To summarize my feeling about the evaluation of materials, there is no technique of evaluation that I know of which is entirely satisfactory*" in his 1971 paper that focuses mainly on the issues of funding blood compatibility studies, but illuminates some of the crucial scientific aspects of the problem.[79] The lack of predictive *in vitro* hemocompatibility tests and the complexities of applying animal results to humans are discussed in both of the hemocompatibility catastrophe papers,[15, 75] as well as a number of other works.[80-82]

In a 1969 article Stoffey et al.[83] stated: "*It would be highly useful to prepare prostheses with the same shapes, using the different methods, and implanting them in the same locations in dogs to give a truly accurate comparison*" – an early expression of the need for standardizing biocompatibility measurements. Today, the topic of standardization remains very high on the list of things that still need to be done.[81, 82]

Some important lessons have nevertheless been learned. Among them is the need to evaluate both the thrombotic and the embolic propensity of the material, as put forward by Kusserov in his seminal 1972 paper.[84] This refers to the need for evaluating processes occurring at the material surface as well as in the fluid phase, or markers that are indicative of both sets of processes (such as platelet consumption).[85-89] Although this issue has been extensively discussed,[15, 75] one continues to find in the literature erroneous parallels between platelet adhesion and material hemocompatibility.

Early on, platelets took the center stage. In retrospect, one could say this foreshadowed the tremendous impact of APT on the success of cardiovascular implants. Although they do not represent the whole story, platelets do occupy the point of intersection of multiple defense and regeneration pathways: thrombosis, adaptive and innate immunity, wound healing, etc. This subject has by now been extensively reviewed.[16, 90-93] Once again, fluid phase and surface measurements are needed to characterize platelet-surface interactions.[80, 86, 89] Platelet activation at surfaces of different materials can be different and materials may be classified according to platelet reactions;[86] what that means in terms of thrombogenicity is not clear. This subject is revisited below.

Another conjecture that appears to have withstood the test of time is the improved performance of hydrophobic coatings/materials. Early observations of vaseline and paraffin coatings on glass extending coagulation time have already been mentioned.[77, 78] Similar *in vitro* observations have been made with PTFE (Teflon)-coated stents evaluated against a panel of parameters.[80] Indeed, perfluorinated polymers appear to top the list; this subject is discussed in some detail in Szott et al.[94] Promising results were also obtained with stents coated with another perfluorinated polymer, poly[bis(trifluoroethoxy)phosphazene] (PTFEP), in *in vitro*[95] and animal studies[96, 97] that led to the first-in-man clinical trials.[98, 99] Limitations of the hydrophobicity conjecture should be kept in mind, however. On one hand, there is the limited success of passive barrier coatings. On the other hand, the relationship between surface wettability and hemocompatibility remains poorly defined, and one would expect it to be affected by coating stability, roughness, topography, and porosity. The mechanisms underlying the superior performance of perfluorinated materials are also not understood. Current hypotheses invoked to explain it center on another conjecture: the relationship between albumin/fibrinogen ratio in the adsorbed protein layer and material thrombogenicity.[94, 100] It should be mentioned that some studies challenge the notion that PTFE is superior to other materials. In particular, Braune et al. demonstrated platelet adhesion and spreading on the surface of PTFE in an *in vitro* study, without, however, considering the state of their activation or surface embolization propensity.[101]

These topics and questions were revisited at the 2014 *BloodSurf* meeting[102, 103] that gave rise to this article. The consensus was that, although important lessons have been learned, we continue to have more questions than answers. The resulting stagnation has translated into loss of funding, declining interest from clinicians no longer expecting breakthroughs, and waning interest from adjacent disciplines, the contributions of which are sorely needed for progress to be made. It is the aim of this article to "shake things up" so that the field can start moving forward again.

3. Is there a need to improve the performance of artificial materials in contact with blood?

Given the existing and evolving therapeutic approaches for managing adverse reactions to biomaterials, is there any remaining need to improve material performance? Indeed, there is.

The key problem with the pharmacological management of material-induced thrombosis is the associated risk of bleeding. Balancing the two entails complex decisions concerning appropriate drug combinations, their dosage and duration, as well as issues of adherence and monitoring. These concerns should be viewed in the context of patient safety and quality of life, as well as sustainability of healthcare systems strained by the ageing populations.

The problem of balancing thrombosis vs. bleeding risks is particularly acute in patients requiring APT and ACT combinations (e.g., aspirin/clopidogrel/warfarin). This so-called triple therapy is used to manage stent-induced thrombosis and the risk of stroke arising from emboli caused by atrial fibrillation or from active cardiovascular implants such as mechanical heart valves and LVADs.[21-23] Holmes et al.[21], Schömig et al.[23], and Paikin et al.[24] discuss the relevant issues in some detail, illustrating the complexity of the decisions facing clinicians. Similar concerns arise when patients on DAPT need to undergo surgery (cardiac or other).[104] Further complexities are introduced by co-morbidities associated with hypercoagulable

and/or proinflammatory states such as diabetes mellitus, obesity, smoking, etc.[56, 105-107] Adherence issues are discussed in refs. [108] and [109]. Disturbingly, 1 to 4% of patients appear to be resistant to APT, developing adverse thrombotic complications, due in part to the variability in responses to clopidogrel among patients.[109-111] This aspect has been recently reviewed.[112]

Heparin-based ACT has its own problems. The use of heparin-coated circuits in CPB, ECMO, and hemodialysis did not alleviate the need for systemic heparin.[18] Complications that are associated with the use of heparin, such as heparin-induced thrombocytopenia,[26] are nothing if not catastrophic, in part because of the scarcity of alternative strategies[19, 113] (although there are new anticoagulants in the pipeline, as discussed earlier.[28, 29, 39])

Furthermore, there are inflammatory complications of artificial materials that are not alleviated by the existing therapeutic strategies. The so-called systemic inflammatory response syndrome in patients after CPB or ECMO is a problem that still awaits a management strategy or, indeed, adequate, systematic investigation.[40, 114, 115].

Last but not least, designing small diameter (< 5 mm) vascular grafts that remain patent following implantation remains a challenge despite the available therapeutic options.[83, 116]

In other words, the performance of blood-contacting devices continues to be limited by thrombotic and inflammatory complications arising from the blood-biomaterial interactions or bleeding complications arising from their therapeutic management. There is plenty to do, and given the scope, seriousness, and significance of the problem, the stakes are high.

4. Solution strategies

Three strategies are proposed below to address the recognized clinical need for minimizing adverse reactions occurring at the blood-biomaterial interface. Their focus is on the specific, biological aspects of the blood-biomaterial interactions. They aim to satisfy two goals: clinicians' immediate need for information about existing materials on one hand, and the long-

term goal of developing systems (material + pharmacological regimen) that minimize the severity and duration of adverse effects on the other. These strategies encompass testing the reaction of blood to biomaterials ("testing"), developing reductionist models for unravelling molecular mechanisms underlying these reactions ("modeling"), and hacking into the cellular communication pathways of the wound healing process with the notion of integrating the implant into them ("hacking").

4.1 Testing

The strategy referred to as "testing" consists of taking existing or newly synthesized materials and evaluating their performance in contact with blood *in vitro*. In the past, this approach focused on a search for the elusive "hemocompatible material". However, since the concept of hemocompatibility has never been properly defined, various surrogate metrics have been adopted in different contexts for evaluating material performance. Most common examples of such metrics included the ability of materials or coatings to resist non-specific protein adsorption and platelet adhesion, or reduce the level of platelet activation in solution in contact with these materials. The relationship between these metrics and the *in vivo* performance of materials remains obscure at best, or, at worst, is entirely lacking. Therefore, echoing several strongly sounded calls,[15, 117] we believe that the wide-spread practice of misidentifying materials as "biocompatible" or "hemocompatible" according to these various arbitrary metrics should stop, because it has led nowhere.

Instead of a search for the elusive "hemocompatible material", we propose to focus testing strategies on uncovering correlations and, subsequently, causal, mechanistic relationships between material properties and the reactions they induce. Recognizing that none of the existing materials are hemocompatible, materials that are currently used in the clinic are a good place to start. Emphasis should be placed on comparing responses between different materials and results between different laboratories. Clinicians will appreciate hard data on what the implant/material is doing mechanistically: is it activating adhering and/or non-adhering platelets, fracturing coagulation cascade proteins at its surface, or giving rise to

complement activation? All three sets of pathways will lead to thrombosis and inflammation, because these systems are linked,[16, 118-120] but the efficiency of the different management strategies might be different depending on which of the systems is activated to which extent, and how antiplatelet agents affect activation levels. Systematic studies addressing these questions are sparse to say the least; they are also often out of reach of any one laboratory and require collaborations between several groups with different capabilities.

Which parameters should be measured *in vitro* to enable meaningful conclusions to be drawn about the performance of different materials in contact with blood? There is currently no *in vitro* test predictive of the material's *in vivo* performance. Therefore, the answer to this question is at the moment partial at best. However, meaningful progress towards a predictive panel of *in vitro* tests can be made by recognizing that (i) these tests have to encompass both the surface phase and the fluid phase reactions; (ii) parameters directly related to thrombogenesis, platelet-, leukocyte-, and complement activation processes need to be measured; and (iii) controls allowing meaningful comparisons between measurements performed in different laboratories and with different materials need to be included. The ISO10993-4[121, 122] gives a starting point with tests for hemolysis, coagulation, platelet, complement, and leukocyte activation; a far more exhaustive discussion can be found in the recent perspective by Braune et al.[81] and a review by Jung et al.[80] that bring into focus the analysis of surface-adsorbed proteins, appropriate controls, blood-drawing procedures, anticoagulant choices, and a well-defined starting point for the testing achieved by characterizing the blood with which the tests are to be performed. Without an adequate characterization of the level of activation of the blood (components) at the outset, a meaningful comparison between results from different laboratories, or even the same laboratory at different times, cannot be made.

Which markers of coagulation, complement, and leukocyte activation should be measured, and under which conditions? There is a relatively well-established set of ELISA assays for measuring thrombin-anti-thrombin (TAT) complex concentration for quantifying thrombin

production, fibrin concentration for quantifying protease activity, β-thromboglobulin (β-TG) and serotonin levels for quantifying the release reactions accompanying platelet activation, PMN elastase for quantifying leukocyte activation, and SC3b and SC5b-9 complexes for quantifying complement activation.[121] They are sensitive, validated, and report the outcomes of both surface and fluid phase processes, without, however, distinguishing between the two. On the other hand, they suffer from long processing times and require relatively large blood volumes. Reducing these takes us into less well charted territory of direct thrombin generation assays[123] and flow cytometry analysis of platelet and leukocyte activation.[124-127] On one hand, these still need to be validated. On the other hand, given recent advances in bead-based flow cytometry analysis of cytokines[128] and the role of platelets in non-hemostatic processes,[91, 93, 120, 129] it may be worth the extra effort and provide a new dimension to the evaluation of platelet activation at biomaterial surfaces. Several studies also point to the importance of quantifying platelet-leukocyte aggregation as a sensitive measure of platelet activation.[130-133] An important note should also be made concerning the flow cytometric detection of microparticles. First, conventional flow cytometers detect ~ 1% of the circulating microparticles.[134] Second, caution should be exercised when using colloidal particles to define size-gates for microparticle detection due to the differences in refractive indexes between microparticles and the colloids.[135] These aspects are reviewed by Shantsila et al.[136] Appropriate combinations of parameters are more likely to provide comprehensive answers than any one of them.

Concerning measurement conditions, several recent reviews discuss different models, without, however, reaching a consensus—except to say that whole blood studies should be performed under shear, if nothing else than to avoid cell sedimentation.[80, 82, 137] The jury is still out as to whether there is one suitable model system. Geometry is important: circular channel cross-section mimics the *in vivo* flow conditions and is more likely to reflect physiological distribution of cells in the flowing blood with the platelets pushed to the periphery.[138-140] The future is most likely with microfluidic systems—because of the small blood volumes used, control, relative ease of standardization, and unmatched possibilities for in situ observation of

adsorption, adhesion, aggregation, and coagulation events. Some information on the subject can be found in recent reviews[141, 142] and several interesting technical papers.[139, 143, 144] One drawback of the microfluidic systems is an increased surface area-to-volume ratio.[80] As of yet, microfluidic technologies have not been tried in the context of biomaterial testing. Of course, for active devices (such as VADs and heart valves), the geometry is specified at the outset.

Finally, materials to be tested also need to be characterized. The need for appropriate surface characterization has been pointed out some time ago.[75] Whereas surface chemistry has traditionally attracted the most attention, it is now clear that topography and surface mechanical properties also play important roles. Specific effects of surface topography and stiffness on cell differentiation have been revealed in a variety of studies: see, for example, reviews by Guilak et al.[145] and von der Mark et al.[146] Particularly noteworthy are recent reports of topography effects on platelet adhesion and activation,[147] on macrophage phenotype switching,[148] and on endothelialisation.[149] In the context of testing, this means that the topography as well as chemistry of the materials tested should be characterized.[150]

Apart from the surface characterization, care should be taken to evaluate the stability of the interface in physiological environments before such a significant investment of effort (see the discussion in ref.[97]). In particular, relatively rapid (months) degradation of poly(ethylene glycol)-based coatings by cultured cells has been reported in the literature.[151] Long-term applications of coatings need to explicitly address the issue of stability, keeping in mind that the internal environment of our bodies is rather aggressive, containing both non-specific and targeted degradation agents such as reactive oxygen species and various enzymes (e.g., proteases) produced in the course of the immune response.

The above discussion should make it clear, that proper *in vitro* testing of biomaterials entails a significant effort that draws on concepts from clinical sciences, natural sciences, and engineering. It also highlights aspects of *in vitro* testing studies that are crucial to maximizing the impact of this arborous effort. It is important to support multi-center or otherwise interdisciplinary studies that incorporate these aspects. This is all the more so since the

cardiovascular implant industry does not appear to be interested in standardization or publically available comparative analysis of implant performance.

Several examples of rigorous *in vitro* hemocompatibility studies can be highlighted.[86, 95, 137, 152, 153] These studies share certain traits: explicit characterization of the starting blood; surface and/or microscopic characterization of the materials; coating stability analysis where relevant; inclusion of fluid-phase and surface-phase reactions as well as testing for multiple pathways (thrombosis, platelet, leukocyte, and complement activation); inclusion of controls. Adherence to the ISO 10993-4 is apparent in the more recent works. Among the drawbacks are limited starting blood and surface characterization in some of the studies, and different controls. However, by and large, there is sufficient information to repeat the studies, provided that identical controls are included.

Stang et al.[152] distinguish between material-induced and pyrogen contaminant-induced reactions, Brubert et al.[153] examine new styrene copolymers with and without heparin coating, as compared to PTFE and bovine pericardium, van Oeveren et al.[137] compare different dynamic testing systems, Mrowietz et al. compare the PTFEP-coated stent with the uncoated stainless steel BMS,[95] while the i*n vitro* study of Haycox et al.[86] is notable both because of the classification of platelet reactions to the different materials and a correlation between their *in vitro* results and *in vivo* observations in a baboon shunt model.

Finally, it has to be mentioned that there is so far no clear way of translating the results of the *in vitro* tests described above to thrombotic failure of a device in the clinic. Ex vivo tests (e.g., the baboon shunt model[85, 154]) fare somewhat better than the *in vitro* tests despite the limited test duration, when evaluated against *in vivo* animal studies.[85] In the end, animal studies remain a necessity. Here, the seminal work by Kusserow (where the evaluation of thrombotic and embolic potential of several polymeric materials is presented) can once again be highlighted.[84]

Animal models have their problems related to the differences between humans and animals in terms of coagulation, inflammation, and wound healing pathways. These include receptor identities, cell counts, and the reactivity of the various pathway components, including in terms of their interactions with material surfaces.[155-161] More subtle effects relate to the different rates of the healing processes in animals and humans and to the fact that animal tests are performed on young, healthy adult animals—a model that may not be suitable for ageing patients whose arteries are affected by atherosclerosis. These issues are discussed in ref. [162] Another interesting review examining various animal models in *in vivo* evaluation of stent performance is that of de Prado et al.,[163] while Carney et al.[164] present an illuminating discussion of animal models for pediatric circulatory support devices.

It would appear that the gamut of currently available tests (*in vitro*, *ex vivo*, or *in vivo*) is better suited for (i) predicting early responses; and (ii) serving a gatekeeper function: failure in these tests is a sign of clinical problems, while the opposite does not guarantee success. This is yet another reason to shift the focus of the testing from the search for the ultimate hemocompatible material to the more basic goal of evaluating physiological responses.

4.2 Modeling

With respect to understanding the mechanisms underlying blood reactions to biomaterials, attempting to emulate physiological conditions becomes a fatal flaw. Such attempts to resolve complexity by emulation result in dogmatic recipes concerning what is and what is not considered to be “physiologically relevant”. In particular, whole-blood studies performed under flow are considered to yield physiologically relevant information, while static or quasi-static study conditions applied to blood or individual blood components are not. This division is both artificial and illogical. There are several reasons for that. Static and quasi-static conditions are common in model studies with blood components, to which we return further below. It should also be remembered, however, that static conditions characterize aneurysms, areas behind venous valves, and the left atrial appendage of the heart. All of these are known thrombi formation and embolization sites (up to 90% of the emboli leading to cerebral strokes

in atrial fibrillation patients are thought to originate in the left atrial appendage; reviewed in ref.[165]). This alone demonstrates the value of static tests. A similar argument applies to wound sites and to interactions with biomaterials in the context of dental- and osteoprosthetics. These implants also come into contact with blood, commonly under stagnant (and hypoxic) conditions. The interactions occurring at their interface with blood influence subsequent integration (or, more commonly, lack thereof) of the device into the wound healing process. While the physiological environments at the different sites are clearly different, the blood components are same.

The second, and perhaps more important, point is that no current *in vitro* measurement mimics physiological conditions for the very simple reason that blood extracted from the body will coagulate if left to its own devices, and very quickly at that. Anticoagulants are used to combat this problem. Different anticoagulants block the coagulation pathways at different points, all the while the action of the other pathways continues unhindered. Case in point: platelets will become activated during storage even in the presence of a citrate anticoagulant over a period of hours,[126, 166-169] while quiescent platelet lifetime in the body is on the order of days. Compounding the problem is the issue of platelet age; a blood sample will contain platelets of different ages, with the associated effects of different activation kinetics and platelet death.[170] Any *in vitro* test is therefore a competition between at least two sets of processes: one set that is related to the material under investigation, and another set that is spontaneous and is a consequence of the removal of blood from its physiological environment. Although it may be supposed that the origin of spontaneous activation of hemostasis is the cessation of the endothelial anticoagulant activity upon extraction, this issue has never been systematically investigated. In part, this is because there is currently no way in which we could collect the blood without inducing material-dependent effects (recall that blood is always collected into a tube, that is also made of a foreign material activating blood components); even cultured endothelial cells may be thrombogenic, as discussed by McGuigan and Sefton[171] in some detail. As a result, the field lacks one of the most important things of all: a negative

control. Despite a significant need, research towards identifying appropriate controls remains underappreciated, because it is often viewed through the prism of the "physiological" recipes.

The third point is that, since blood and its components are only useful for a very short period of time outside the body, *in vitro* tests can only be performed over time periods of minutes to hours. Yet, these tests need to predict the behavior of materials and devices over time periods of months to years. On the other hand, short-term (minutes) exposure of materials to concentrated protein solutions (such as plasma) renders them non-interactive—this is the basis for blocking reactive surfaces with concentrated albumin solutions used in molecular and cell biology assays. The apparent paradox may be resolved by considering the timescales on which the remodeling and chemical modification of the adsorbed protein layers exposed to complex protein mixtures occurs. This, once again, is an area which remains largely unstudied.

Finally, physiological and pathophysiological conditions differ from each other. For example, it was shown in a recent study that using platelets from patients suffering from coronary artery disease vs. platelets from healthy subjects in a static thrombogenicity tests system led to significantly different results in platelet adherence and activation.[101]

The path out of these woods may be found through model studies that entail basic physics and physical chemistry experiments applied to blood (or blood components) under controlled conditions. They should take into account the relevant time- and length scales, and are aimed at identifying *minimal sets of elementary events and their rates that are capable of describing various phenomena observed in practice*. The relevant phenomena include spontaneous coagulation of blood outside the body and the way the rate of this process depends on time, temperature, and protein adsorption to the surface of the container; differences in platelet activation by different surfaces; the relationship between platelet activation and spreading; the relationship between adsorbed protein conformation and platelet activation; etc. These model studies require certain levels of abstraction, reducing complex phenomena to sets of elementary components and interactions between them. Therefore, the question asked of these studies should not be whether they do or do not emulate

a physiological situation, but whether they have identified, in a causal manner, mechanisms underlying particular aspects of the behavior of the system (material + blood (components)). Physiological relevance should then be established by examining the manifestations of these mechanisms in the appropriate *in vivo* or *ex vivo* systems.

A discussion of modeling physiological phenomena would be incomplete without mentioning the limitations of the reductionist approach applied to complex systems.[172, 173] Indeed, coagulation, inflammation, and complement, are examples of interconnected biological networks ripe for systems biology approaches.[174, 175] In this context, we see the objective of the modelling studies not in identifying any one particular factor responsible for adverse reactions to biomaterials, but in revealing hidden interactions and degrees of freedom at the blood-biomaterial interfaces by examining them under (apparently) unusual sets of conditions. These degrees of freedom may be hidden because they occur on inaccessible timescales under physiological conditions, or because we do not yet know what their physiological roles are. Dismissing these degrees of freedom as artifacts limits our ability to discover new physiological and pathophysiological phenomena and understand their mechanisms. This serves no-one—not the scientist and not the patient.

One example of such model systems is “purified” platelets. Separated from the protein-rich matrix of the blood plasma, purified platelets are clearly as far as one can get from a physiological situation of whole blood while retaining the basic structure of the platelets themselves. As such, observations with purified platelets tend to be viewed with suspicion. One should recall, however, that several important signaling cascades in the platelets incorporate plasmatic components that mediate platelet-platelet interactions. The most common example is that of ADP-stimulated platelets: stimulation leads to aggregation that leads to thromboxane production. Thromboxane triggers other platelet responses typically associated with platelet activation, such as dense granule secretion, irreversible aggregation, etc.[176-178] Purified platelets therefore allow platelet signaling pathways to be investigated independently of the plasmatic feedback loops, revealing sets of responses that are controlled

independently. In the same vein, adsorbing purified platelets on glass immediately reveals the existence of subpopulations with respect to the expression of phosphatidyl serine and the activated form of GPIIb/IIIa.[179] These subpopulations appear similar to the ones identified *in vivo* by intravital microscopy and *in vitro* by flow cytometry,[180] offering a way to study platelets in these subpopulations separately. Moreover, experiments with purified platelets reveal differences in the way surfaces activate platelets,[181, 182] as do experiment with platelet-rich plasma,[183, 184] and whole blood[86]—once again showing that claims of the "non-physiological" nature of static experiments with purified platelets do not stand up to scrutiny.

Another example where models offer a unique insight into the mechanism underlying complex biointerfacial phenomena concerns the effect of adsorbed protein conformation on surface-platelet interactions. It has been known for some time that adsorbed protein conformations depend on protein concentration in solution during adsorption.[185] Specific deviations in the secondary structure of, e.g., fibrinogen upon adsorption have been revealed by circular dichroism relatively recently: the structure of the adsorbed protein is near-native (mostly α-helical) when it is allowed to adsorb at a high protein concentration but deviates significantly (becoming predominantly β-sheet) when it is allowed to adsorb at a low protein concentration; the degree of adsorption-induced unfolding (as measured by % loss in α-helicity) was found to correlate with increased platelet adhesion.[186] It would be very interesting to examine how these changes in protein conformation are related to platelet activation profiles, which are now known to be different on different surfaces and under different conditions, and to examine the nature of the structural elements responsible for the observed effects. Without a doubt this will offer new ways of regulating platelet-surface interactions, possibly even leading to the identification of new drug targets on the platelet surface. In the context of the earlier discussion on timescales, there is evidence that these short time (minutes) experiments offer a glimpse of what will happen with proteins and platelets at material surfaces over months, with the potential occurrence of aging-induced conformational changes of the adsorbed proteins leading to a time-dependent platelet response.[187]

Although these insights into adsorbed protein conformational changes and their effects are relatively recent, it has been widely observed and is well established that the composition of the protein layers adsorbed from blood directs subsequent cellular reactions to foreign materials.[16, 42, 188-191] A protein layer covers the surface of a blood-contacting material within seconds, unless the surface is purposely designed to prevent protein adsorption. Resistance to protein adsorption is imparted on surfaces by modifying them with hydrophilic species such as poly(ethylene glycol),[192] zwitterionic species such as phosphorylcholine[193] or sulfobetaine,[194] or, more recently, omniphobic tethered liquid perfluorocarbon coatings.[195] Long-standing efforts in this direction have yielded approaches for knowledge-based design of interfaces with specifically engineered properties[196-198]—a feat impossible without reducing non-specific adsorption. These efforts have also greatly benefited other fields.[199, 200] Perhaps the recently introduced materials could aid in the design of short-term devices (catheters and CPB circuit components) by alleviating the limitations of the previous approaches to achieve protein resistance.[201, 202] However, it should be recalled that vessel endothelium has multiple mechanisms for keeping coagulation and inflammation in check. Attempts to design interfaces mimicking these multiple mechanisms have appeared in the literature.[42, 196, 198] Indeed, given past experience, the non-specific barrier approach that favors lack of interaction at the blood/biomaterial interface over active integration or participation in the inhibitory pathways, may not be the right direction for improving the hemocompatibility of long-term implants.[87, 88, 203]

An equally well-established notion is the dynamic nature of adsorbed protein layers.[204, 205] On the other hand, the effect of surface chemistry on the composition adsorbed protein layers remains obscure, and this is another area where model studies can yield essential insights.[206, 207] Given that certain proteins are believed to be desirable (e.g. albumin as a passivating element) and others undesirable (e.g. fibrinogen as a platelet-reactive element),[100] interface design based on knowledge of adsorbed protein layer properties and how they influence biological functions may be a fruitful approach. However, the efficient exploration of this approach depends on having much deeper knowledge of the relationship between surface

properties, adsorbed protein layer properties, and subsequent adverse events than is currently available. Here, studies of nanoparticles in contact with blood or plasma, where a so-called "protein corona" is formed, are beginning to generate extensive detailed data on the composition of the corona.[208-210] In this context Chan et al. have called for the development of a database "…for nanoparticle-serum protein interactions" to allow correlations between material properties and biological response, remarking that "A concerted global effort is required to build this database."[211] This would be an equally laudable goal for the blood compatibility community.

The above examples illustrate how model studies can reveal phenomena hidden by the complexities of the real systems. This aspect is out of the reach of attempts to emulate the complexities of the pathological or physiological situations in the most exact way without considering the underlying mechanisms. While *in vivo* studies are essential for evaluating the performance of specific design concepts, *in vivo* conditions are so complex that it may be impossible to ascertain the cause of an observed response or to identify directions to be taken for improving a system. In contrast, controlled *in vitro* studies, by virtue of the simplified conditions, allow specific cause-and-effect relationships to be elucidated and the underlying mechanisms unraveled. Both approaches have their value and their place.

4.3 Hacking

The above discussion of hidden degrees of freedom brings us to the last of the three strategies proposed as the way out of the blood compatibility catastrophe. Its essence is that cells "talk", and we should learn to "listen". Hemostasis and inflammation are defense systems that evolved to maintain the integrity and functionality of the organism. Under normal physiological conditions, initial hemostatic and inflammatory responses are taken over by regenerative mechanisms orchestrated and carried out by the same players: platelets, leukocytes, and their regulatory mediators. The ultimate goal of blood compatibility studies should then be to harness these innate regenerative mechanisms and integrate the implants into their regulatory circuits. Here, the regenerative functions of platelets come to mind,[91, 212-

[215] as well as the broader context of the fibrinolytic system, leukocytes, fibroblasts, and endothelial (progenitor) cells. Moreover, while the idea itself is not altogether new—it has been known for a very long time that humans have a limited ability to endothelialize implants[83, 216, 217]—recent advances in understanding cell signaling, the knowledge that cell fate can be controlled by surface geometry[148, 218] and other material factors,[219] may allow a fresh look at this old problem. It is becoming more and more clear that successful implant surfaces will be dynamic, capable of reorganizing or regenerating on some timescale—at a minimum, the timescale sufficient for endothelialisation. On medium time scales (hours to days), an appropriate response can be engineered into an interface by combining resistance to non-specific protein adsorption with specific desirable reactions.[196-198] The notion that the next breakthrough is likely to come from the understanding of the molecular repair mechanisms, and how they are affected by an implant, has been recognized by scientists working at the bio/non-bio interface.[189, 220] This territory continues to remain largely uncharted and presents ample opportunities for innovation.

5. Conclusions

The take-home message from this discussion is that we need to take a step back in order to move forward; to shift the focus from chasing the holy grail of finding a perfectly hemocompatible biomaterial to the basic biological questions surrounding blood-biomaterial interactions that remained unanswered for many years. It is suggested here that this could be achieved through a combination of the existing testing approaches with appropriately designed reductionist models, provided these are conscious of the relevant biological (signaling) context. The focus on these questions will lead to the generation of falsifiable or verifiable hypotheses concerning factors that make surfaces hemocompatible. There are past examples of systematic studies doing exactly that in the literature. This approach has a chance of leading to successful bioengineering solutions, while the usefulness of indiscriminant

testing of materials against poorly defined metrics is limited. In the meantime, researchers should aim to provide to clinicians tangible results in terms of the mechanisms that are at work at the implant surface. This will aid in the improvement of the existing and the design of new therapeutic regimens.

Conflict of interest statement

None.

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