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Shear-induced platelet adherence and activation in an *in-vitro* dynamic multiwell-plate system

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Abstract

Circulating blood cells are prone to varying flow conditions when contacting cardiovascular devices. For a profound understanding of the complex interplay between the blood components/cells and cardiovascular implant surfaces, testing under varying shear conditions is required. Here, we study the influence of arterial and venous shear conditions on the *in vitro* evaluation of the thrombogenicity of polymer-based implant materials.

Medical grade poly(dimethyl siloxane) (PDMS), polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE) films were included as reference materials. The polymers were exposed to whole blood from healthy humans. Blood was agitated orbitally at low (venous shear stress: 2.8 dyne·cm^{-2}) and high (arterial shear stress: 22.2 dyne·cm^{-2}) agitation speeds in a well-plate based test system. Numbers of non-adherent platelets, platelet activation (P-Selectin positive platelets), platelet function (PFA100 closure times) and platelet adhesion (laser scanning microscopy (LSM)) were determined.

Microscopic data and counting of the circulating cells revealed increasing numbers of material-surface adherent platelets with increasing agitation speed. Also, activation of the platelets was substantially increased when tested under the high shear conditions (P-Selectin levels, PFA-100 closure times). At low agitation speed, the platelet densities did not differ between the three materials. Tested at the high agitation speed, lowest platelet densities were observed on PDMS, intermediate levels on PET and highest on PTFE. While activation of the circulating platelets was affected by the implant surfaces in a similar manner, PFA closure times did not reflect this trend.

Differences in the thrombogenicity of the studied polymers were more pronounced when tested at high agitation speed due to the induced shear stresses. Testing under varying shear stresses, thus, led to a different evaluation of the implant thrombogenicity, which emphasizes the need for testing under various flow conditions. Our data further confirmed earlier findings where the same reference implants were tested under static (and not dynamic) conditions and with fresh human platelet rich plasma instead of whole blood. This supports that the application of common reference materials may improve inter-study comparisons, even under varying test conditions.

1. Introduction

The standard ISO 10993-4:2017 (Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood) provides a set of principles for hemocompatibility testing of e.g. polymer-based biomaterials [1–3]. However, for a profound understanding of the complex interplay between material surfaces and blood components/cells, further testing is required to characterize thrombogenic properties of materials sufficiently [4–7]. Static tests - performed under no or low flow conditions - using platelet rich plasma or whole blood are commonly applied [8,9]. However, cardiovascular implants, which are in contact with the flowing blood, should be tested additionally under dynamic conditions with shear stresses similar to those occurring *in vivo* [10–12]. In this study, the influence of different agitation speeds and associated shear stresses on the activation and adherence of platelets on polymeric implant materials were evaluated using a readily accessible and reproducible dynamic test system.

2. Materials and methods

2.1. Blood sampling, characterization and preparation

The study was designed in accordance with the ethical guidelines of the journal and received an approval of the ethics committee of the Charité University Medicine Berlin (EA2-018-16) [13]. Sodium citrated whole blood (1 mL anticoagulant + 9 mL blood, 0.106 $mol \cdot L^{-1}$ final sodium citrate concentration) was obtained from apparently healthy humans (n = 6 in total). Blood donors were included in the study in case they had not taken any medication that is reported to influence platelet function, coagulation or the immune response (at least 10 days). The donor blood was characterized prior any testing as previously described [14]. Briefly, demographic data, blood hemogram values, platelet function as well as acute inflammatory markers (e.g. C-reactive protein levels) were tested. Donors were excluded from the study in case values were not within reference ranges for healthy humans, early inflammatory processes and abnormalities in platelet count/function were recorded. The blood samples were allowed to rest prior any further testing (15 minutes). All tests were completed within four hours after blood donation to ensure appropriate platelet functionality.

Table 1. Physicochemical characterization of the tested polymers (Reprinted from [15], Copyright 2017, with permission of Elsevier).

Polymers	**Root mean square surface roughness (R_q)**	**Advancing water-air contact angle (ϑ_{adv})**
Poly(dimethyl siloxane)	14 nm ± 2 nm	109° ± 2°
Poly(ethylene terephthalate)	24 nm ± 2 nm	79° ± 1°
Poly(tetrafluoro ethylene)	62 nm ± 6 nm	119° ± 2°

2.2. Material substrates

Three polymer-based implant materials were included in the study: Poly(dimethyl siloxane) (PDMS, Bess Medizintechnik GmbH, Berlin, Germany), Polyethylene terephthalate (PET, Nalge Nunc International, New York, USA) and Poly(tetrafluoro ethylene) (PTFE, Bess Medizintechnik GmbH, Berlin, Germany). Root mean square surface roughness and advancing water-air contact angles were obtained as previously described and characterize the surface properties of the different polymers (see Table 1) [15].

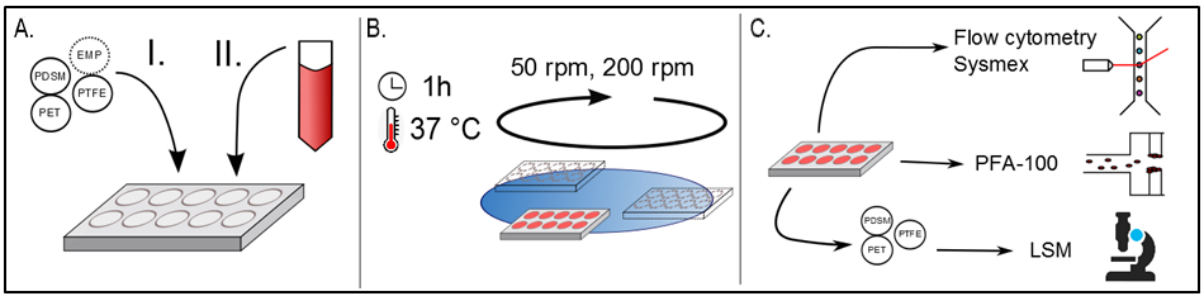

Figure 1. Schematic of experimental setup and data collection. A. Placing of polymer samples in well plate (I) and filling with whole blood after equilibration with phosphate buffered saline (II). B. Agitation of the well plates at two different shear conditions (50 rpm or 200 rpm). C. Analyses of the whole blood samples after 1 hour agitation (whole blood cell count (SYSMEX), flow cytometry (CD62P positive platelets) and platelet function analysis (PFA)) as well as of the surface adherent cells (Laser Scanning Microscopy (LSM)).

2.3. Dynamic test setup

The polymers were placed in a 24-well suspension culture plate (CELLSTAR®, Greiner Bio-One, Frickenhausen, Germany) and equilibrated with phosphate buffered saline. Samples were divided into

two groups: a high (200 rpm) and low agitation speed group (50 rpm). A test system filled with blood but without a test material served as agitated control (EMP). The samples were agitated on an orbital shaker and exposed to whole blood for one hour at 37 °C (Figure 1) [16]. Each group was measured in duplicates for each donor (n = 6 donors in total). The shear stress developed for a liquid exposed to orbital agitation can be estimated by the equation shown below [17]. For the applied agitation speeds, shear stresses were: 0.28 $N \cdot m^{-2}$ (2.8 $dyne \cdot cm^{-2}$) and 2.22 $N \cdot m^{-2}$ (22.2 $dyne \cdot cm^{-2}$), respectively.

$$\tau_w = a \cdot \sqrt{\rho \cdot \eta \cdot (2 \cdot \pi \cdot f)^3} \quad (1)$$

a = radius of gyration,
ρ = density of the fluid,
η = dynamic viscosity of the fluid,
f = frequency of rotation [Hz].

2.4. Non-adherent and adherent platelets

Non-adherent blood cells were evaluated using a SYSMEX haematolyzer (Sysmex XS-800i, SYSMEX, Norderstedt, Germany). Platelets adherent on the polymer sample surface were stained with buffered glutardialdehyde (2 wt%, 30 min, room temperature). Polymers were embedded (e.g. Mowiol 4–88 mounting medium, Carl Roth GmbH, Karlsruhe, Germany) and covered with glass coverslips (Menzel GmbH, Braunschweig, Germany). Image acquisition was carried out with a confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany). ImageJ software (National Institutes of Health, USA) was applied to analyze surface adherent blood cells.

2.5. Platelet function

Platelet function was assessed with the Siemens PFA100® device (Siemens Healthcare, Erlangen Germany). Collagen and ADP coated cartridges were applied for measuring closure times after exposure of the polymer samples to whole blood. The percentage of activated (CD62P - P-Selectin - positive) platelets was determined by co-labeling with anti-human CD42a and CD62P (P-Selectin) antibodies. Flow cytometry measurements of whole blood samples and data analysis were conducted utilizing a MACSQuant Analyzer 10 (Miltenyi Biotech, Bergisch Gladbach, Germany).

2.6. Statistics

Values of the six donors included in this study are given as arithmetic mean ± standard deviation. Handling and measurements were randomized, in order to reduce systematic errors. The statistical analyses comprised one factorial ANOVA and Dunn's tests for multiple comparisons. P-values less than 0.05 were considered significant. All statistics were compiled in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California USA).

3. Results

3.1. Non-adherent and adherent platelets

For both agitation velocities, highest counts of non-adherent platelets were observed in the empty test system. Values obtained at 200 rpm were lower compared to those obtained at 50 rpm (Figure 4A). While highest platelet counts were observed for PDMS, values were intermediate for PET and lowest for PTFE. Also differences between these samples were more pronounced after 200 rpm agitation, however, those measurements revealed higher variations. The mean platelet volumes (MPV) of all samples were slightly increased after 200 rpm agitation. Differences between PDMS and PTFE were significant after 50 rpm agitation only (Table 2). In contrast, the ratios of large platelets (PLCR) were considerably decreased after 200 rpm compared to 50 rpm agitation and showed the same significant difference between these material samples (Table 2). Platelet distribution widths (PDW) were increased for all polymers after 200 rpm agitation and revealed considerable stronger differences between the polymers (e.g. PDMS: 129.2 fL ± 23.0 fL and PTFE: 137.8 fL ± 13.2 fL) than values obtained under 50 rpm agitation. Differences were significant between PET and PTFE for these conditions only. In accordance with the decrease of circulating platelets, numbers of cells adherent on the polymer surfaces were clearly increased for PET and PTFE when tested at higher shear forces (200 rpm agitation speed, Figures 2 and 3B). Differences between PDMS and PTFE were significant, however, measurements for 200 rpm revealed higher standard deviations than those obtained for the low agitation speed (Figure 4B).

Table 2. Indices of circulating platelets

Parameter	**Unit**	**50 rpm (2.8 dyne·cm^{-2})**				**p**
		EMP	PDMS	PET	PTFE	
Mean platelet volume	fL	94.8 ± 3.5$^{\#}$	94.7 ± 3.1$^{\#}$	97.0 ± 1.9	98.67 ± 3.9	<0.05
Platelet distr. width	fL	110.8 ± 3.8	105.3 ± 5.2$^{\#}$	113.2 ± 4.6	115.3 ± 7.5	<0.05
Platelet large cell ratio	%	223.3 ± 24.42	218.0 ± 25.64$^{\#}$	238.5 ± 14.9	248.0 ± 24.43	<0.05
Parameter	**Unit**	**200 rpm (22.2 dyne·cm^{-2})**				
Mean platelet volume	fL	100.0 ± 2.2$^{\#}$	105.8 ± 6.6	105.3 ± 4.8	105.8 ± 2.0	>0.05
Platelet distr. width	fL	115.8 ± 6.2	129.2 ± 23.0	127.8 ± 17.4	137.8 ± 13.2	>0.05
Platelet large cell ratio	%	255.3 ± 14.14$^{\#}$	298.3 ± 51.0	300.0 ± 38.2	308.7 ± 17.2	<0.05

(Data are given as mean ± standard deviation, p values indicate statistical differences, * = sign. different to PDMS, + = sign. different to PET, # = sign. different to PTFE).

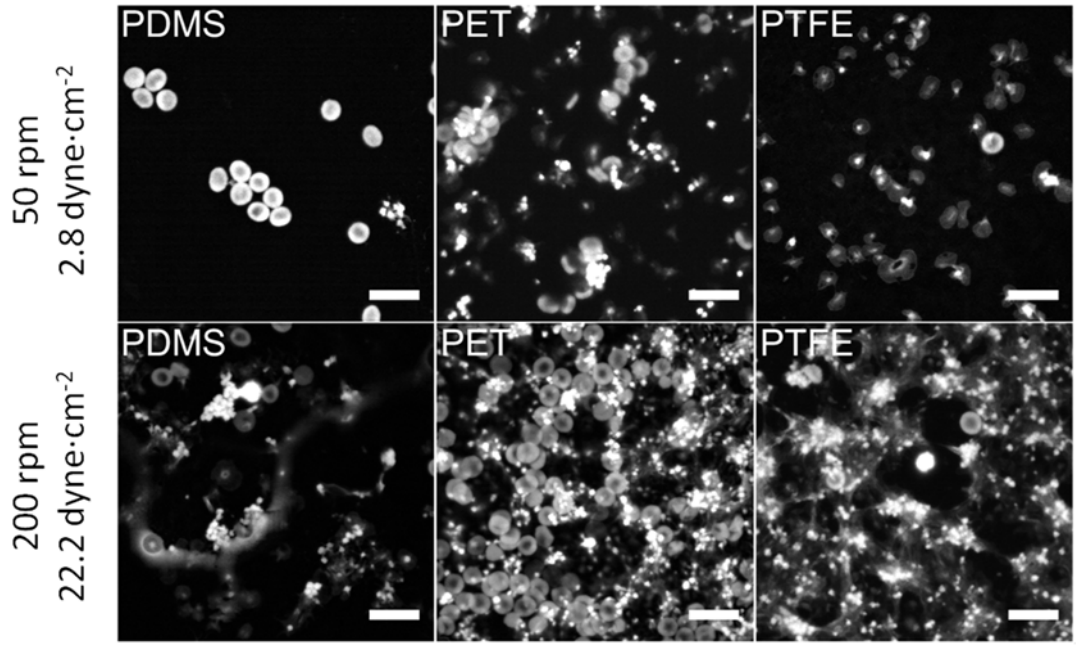

Figure 2. Representative confocal laser scanning images at 100-fold primary magnification of each substrate (PDMS, PET, PTFE) and each agitation speed; upper row: 50 rpm, lower row: 200 rpm. Scale bar = 20 µm.

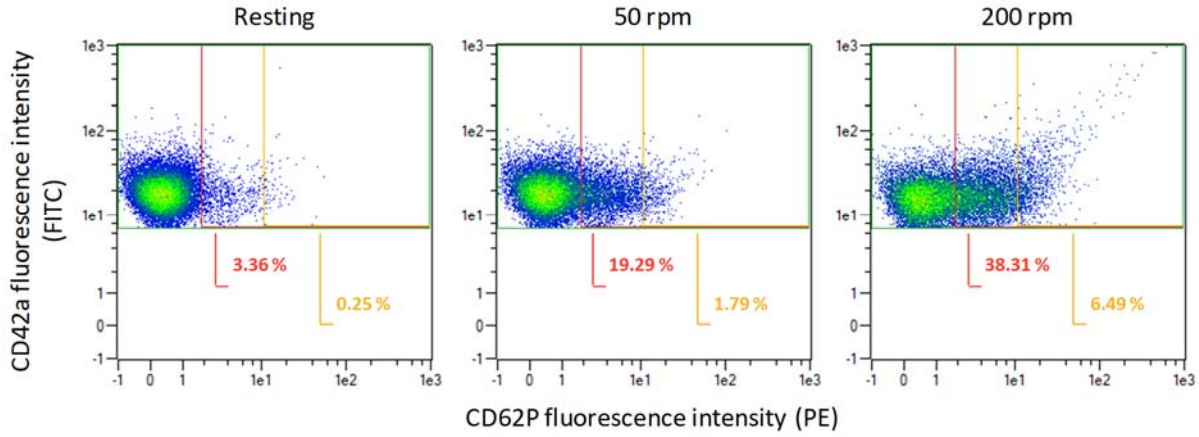

Figure 3. Representative flow cytometry data of one donor (approx. 21,000 $CD42a^+$ events are displayed per measurement) and gating strategy of resting platelets and samples from the empty test system after 50 rpm and 200 rpm agitation. Pseudocolor density plots display $CD42a^+$ events (green gate) as well as percentages of CD42a/CD62P positive events (red gate) and CD42a/CD62P aggregates (yellow gate) at resting conditions, 50 rpm and 200 rpm agitation speed (for the sake of clarity, CD42a negative events are not shown).

3.2. Platelet function

Compared to values for 50 rpm, the amounts of platelets expressing CD62P (P-Selectin) were increased for the empty test system and all polymers tested at 200 rpm (Figure 4C). As shown in Figure 3, flow cytometry data further revealed a shear stress dependent increase of CD42a/CD62P double positive events with increased mean fluorescence intensity, which were interpreted as platelet

aggregates. At 200 rpm, values decreased in the same manner as reported for the circulating platelets (PDMS>PET>PTFE), while no trend was observed for 50 rpm. Variances were in a similar range for all samples analyzed. Similar differences between the agitation velocities were observed for the PFA100 closure times (Figure 4D). According to the data of the CD62P expression, closure times for the empty test system differed. The values for 50 rpm and 200 rpm showed no clear trend and no significant differences between the polymer samples were observed. Compared to closure times obtained at 50 rpm, variations were stronger at 200 rpm agitation for all measured samples and the empty test system.

Hemolysis was not detected for any of the samples analyzed.

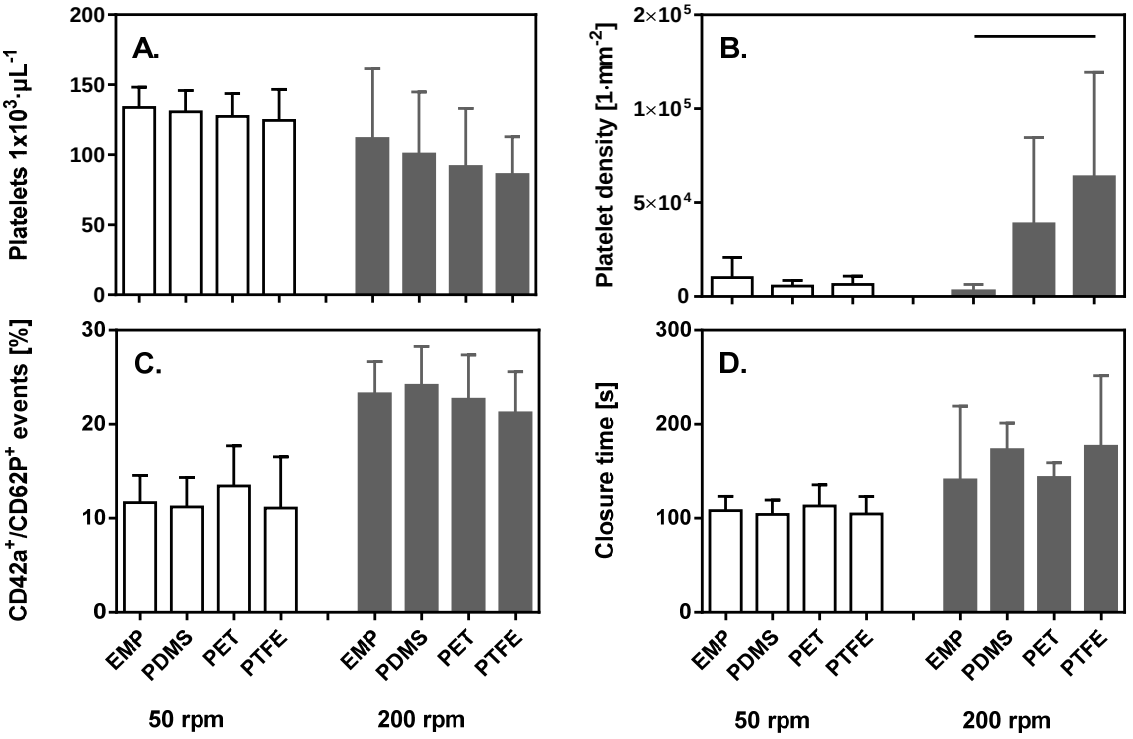

Figure 4. Analyses of circulating and material surface adherent platelets as well as platelet function. A) Quantification of non-adherent blood cells. B) Image-based quantification of adherent platelets. C) Expression of CD62P on CD42a positive platelets (Flow cytometry data, $CD42a^+/CD62p^+$ events). D) Analysis of platelet function in the Platelet Function Analyzer (PFA100). Data are given as mean ± standard deviation. Horizontal black bars indicate a statistical significant difference ($p < 0.05$).

4. Discussion

In this study, we introduced a readily accessible well-plate based dynamic whole blood test system and studied the influence of shear stresses on the interaction of platelets with polymeric implant

materials. The applied shear stresses of 2.8 dyne·cm^{-2} (at 50 rpm) and 22.2 dyne·cm^{-2} (at 200 rpm) were within the physiological range of normal veins (1 – 6 dyne·cm^{-2}) and normal arteries (10 – 70 dyne·cm^{-2}) [18,19].

In the empty test system, the arterial shear stresses at 200 rpm led to an increase in platelet activation and aggregation. The numbers of circulating platelets, the platelet indices and the proportions of CD62P positive platelets were, thus, less influenced when the lower venous shear stress was applied at 50 rpm agitation speed (see Figure 4 and Table 2). These results correspond well with earlier findings of Ikeda and coworkers, who showed that platelet activation and aggregation can be observed at shear stresses of about 12 dyne·cm^{-2} in sodium citrated blood samples, although, normal time-average levels of fluid shear stress in the arterial circulation are approximately 20 dyne·cm^{-2} [20,21].

Despite the shear induced pre-activation of the circulating cells, differences in the interaction of the platelets with the material surfaces were more pronounced under high than under low shear conditions. Most apparent differences were observed in the reduction of the circulating (non-adherent) platelets and the numbers of platelets adherent on the polymer surfaces (see Figures 2, 4A and 4B). The results revealed a strong interaction of the platelets with PTFE, an intermediate interaction with PET and the lowest interaction with PDMS. These results confirm earlier data on platelet adhesion and activation, which were obtained for the same reference materials and anticoagulation but under static test conditions and utilizing fresh human platelet rich plasma [15,22].

Beyond platelet adhesion, also function of the circulating platelets was affected differently by the polymer surfaces under the elevated shear conditions. Particularly the CD62P positive events showed a clear trend for the three tested polymers: PDMS>PET>PTFE (Figure 4C). This decrease was directly related with the decreasing numbers of non-adherent platelets (PDMS>PET>PTFE), inversely related with the increasing platelet large cell ratio and, particularly, with the increasing number of adherent platelets (PDMS<PET<PTFE). These results indicate that the activated platelets mainly interacted with the material surfaces and might have been reduced in the circulation. As indicated by the platelet indices and flow cytometry data (Table 2, Figure 3), platelet aggregate formation was elevated with increasing shear stress and thrombogenicity of the material surfaces. Taking this into account, the observed decrease of the number of circulating CD62P positive events appears plausible. The increased consumption of activated platelets by the material surface and the platelet aggregation very likely resulted also in a reduced availability of platelets capable of closing the aperture of the PFA device and, thus, induced the prolonged closure times observed at higher shear stresses (Figure 4D). These findings are in agreement with earlier reports about prolonged PFA closure times in case of

significant reductions in platelet counts (< 100 x 10^9 platelets · L^{-1}) and non closure in cases of severe thrombocytopenia (e.g. < 10 x 10^9 platelets · L^{-1}) [23–25]. Despite PFA closure times were considerably elevated after contacting PDMS and PTFE under these conditions, no clear trend was observed that was related to the other parameters. We concluded that in a closed test system with a relatively high material surface to blood volume ratio and under the conditions of platelet activation and consumption on test material surfaces, the PFA data revealed less sensitivity to distinguish between the polymers than CD62P flow cytometry data. The increased variability of the data obtained at the higher agitation speed, very likely reflect the donor variability. Best et al. for instance reported that donor specific levels of VWF show a stronger correlation to varying PFA-100 closure times than GPVI receptor densities, which did not affect platelet function under high shear [26,27]. This is in good agreement with our data, showing higher donor variability between the two agitation speeds for PFA closure times and interaction of platelets with the material surfaces but not for the densities of CD62P positive platelets.

An advantage of the introduced assay lies in its readily accessibility, particularly since the applied disposable parts and devices are typically available in a standard biological/clinical laboratory. More sophisticated and complex test systems might mimic physiological situations more appropriately. However, approaches that allow other laboratories to reproduce tests more easily represents an effort necessary for improving the comparability of data from different studies and laboratories [28,29]. A further advantage is the well-plate based approach, which allows testing of different material samples simultaneously with fresh blood from the same donor while reducing donor to donor variability [30]. The test systems can be placed in a regular cell culture CO_2 incubator, which enables keeping the environmental gas composition constant and reducing the influence of changes on blood pH during the incubation [31]. On the other hand, the open well architecture represents a disadvantage of the test system. The air liquid interface might induce aggregation and conformational changes of blood plasma proteins as well as activation of the contacting blood cells [32–34]. Overall, our data revealed that this is influencing the studied parameters far less than the applied shear forces, since platelet adhesion and activation was substantially reduced when samples were agitated at lower shear forces, and hemolysis was not observed at all.

5. Conclusion

Mimicking venous and arterial shear stresses to simulate how platelets interact with polymeric implant materials at different shear conditions lead to a substantial different evaluation of the thrombogenicity of the tested polymeric implants. For the majority of the parameters studied here, a stronger discrimination between the polymers was shown when higher shear stresses were applied. The latter resulted in a considerable pre-activation of the platelets under these test conditions. Results obtained in this dynamic whole blood study confirm earlier findings where the same set of polymeric implants was tested with platelet rich plasma under static conditions. Both tests reveal a low thrombogenicity of PDMS, an intermediate of PET and strongest platelet activation and adhesion on PTFE [15,22]. This work emphasizes the importance of testing biomaterial thrombogenicity *in vitro* under different physiological flow conditions [35]. It further shows that utilizing harmonized reference materials can improve the inter-study comparability even when tests are conducted under varying conditions, such as static or dynamic conditions and single cell suspensions or whole blood.

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