

Assessment of hemocompatibility of materials with arterial blood flow by platelet functional tests

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Abstract. Hemocompatibility test of medical materials aims to detect adverse interaction between artificial surface and blood, which can activate or destruct blood components. In arterial flow conditions, due to a high shear stress, platelet is the cell critical for the hemocompatibility compliance. A classical instrumentation for the dynamic test of hemocompatibility involves a flow chamber with a contact surface between blood stream and tested plate. In the current study we investigated a simplified model of the whole blood shear stress, based on a cone and plate rotational viscometer. Several indices of platelet activation were analyzed, including platelet- and granulocyte-platelet aggregates, platelet activation markers and platelet-derived microparticles. This model allowed to estimate platelet destruction, however no adhesion could be measured directly. In following tests of several polymer and metallic layer coated materials, the test revealed comparable performance to more laborious hemocompatibility experiments. We suggest, that thrombogenic potential of platelet-derived microparticles, which can be accurately measured in blood plasma, offers very useful estimate of hemocompatibility. Moreover, this parameter has already been validated in clinics and could be used for monitoring of implanted cardiovascular materials.

Key words: hemocompatibility tests, blood shear stress, platelet microparticles.

1. Principles of tests for hemocompatibility of medical materials

International Organization for Standardization (ISO) developed a guidance on testing medical materials that have contact with circulating blood (ISO 10933-4). In brief, a hemocompatible material must not adversely interact with any blood components [1]. A measure of this adverse interaction can be inappropriate activation or destruction of blood components. The guidelines provide a structured selection of methods useful in testing for interactions with blood. It is based on the intent to use of the material, however, it does not provide the exact test methods or evaluation criteria. Instead, a list of various applicable references is suggested.

Blood is a complex tissue, comprising plasma and cells. Blood plasma is the isotonic electrolyte solution corresponding to 0.9% sodium chloride, with relatively high protein content (40–60 g/L). Albumin makes more than half of total plasma proteins, but many low level constituents are critical for the function of whole organism, e.g. clotting factors, anti-proteases, transporter proteins, or immunoglobulins. The most abundant blood cells are erythrocytes, which extremely high count of 4 million per one μL causes that 40% total blood volume is occupied by erythrocytes. These cells are also the most rigid ones and prone to rupture and subsequent hemolysis, which can also occur upon the change in osmotic pressure, or interaction with mechanical devices. Blood platelets are roughly twenty times less abundant and platelet diameter is only the one fifth of erythrocyte. Blood platelets are critical for vascular homeostasis, as they easily activate in contact with the exposed components of the vessel wall, grant-

ing protection for vascular bed integrity and sealing of any blood leaks. This primarily hemostatic function of platelets could lead, in certain conditions, to the thrombosis. It is also one of the major obstacles in the use of biomaterials that contact with blood. Hemocompatibility categories pointed out by ISO guidelines refer to several aspects of blood physiology, as: coagulation, platelet status, thrombosis, hemolysis and activation of innate immunity complement proteins. Thrombosis occurs normally only at site of vascular injury and involves platelet adhesion followed by the activation of blood clotting cascade and formation of fibrin clot. Though blood platelets are a major morphotic component of thrombus, other cells, like erythrocytes and leukocytes can also accumulate at the site of fibrin deposition. Activated leukocytes easily adhere to the surface of endothelial cells, and migrate out of bloodstream into surrounding tissues. This property is also important for testing of hemocompatibility, as these cells can activate alongside the platelets and adhere to the vascular wall promoting thrombosis.

Commonly used test conditions mimicking vascular flow aim to simulate dynamic interaction between whole blood and biomaterial [1–3]. Dynamic test differs from a static interaction by introduction of shear forces activating blood cells. Shear stress, which is measured in units of pressure, is expressed more conveniently as the shear rate, which changes linearly with the stress, providing constant viscosity of blood. From technical point of view, materials are tested using flow chambers of a different design (Fig. 1). Blood used in these tests is anti-coagulated with sodium citrate or heparin to prevent clotting. To avoid artifacts, dynamic tests for hemocompatibility are preferably performed using one-pass of blood

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and their duration ranges usually from 2 to 5 minutes but can be extended up to 1 hour.

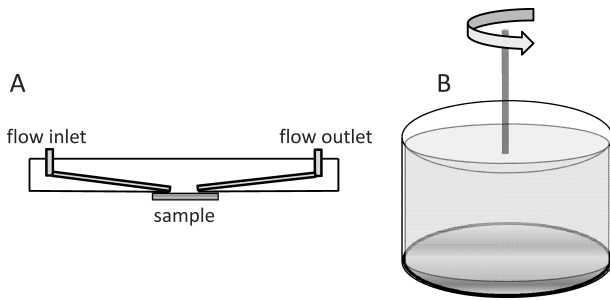


Fig. 1. A typical flow chamber (A) and cone and plate analyzer (B)

1.1. A simplified method to assess hemocompatibility in high shear stress conditions. The study was designed to evaluate performance of an alternative *in vitro* dynamic test for hemocompatibility. Instead of a linear flow chamber, the test is based on the cone and plate analyzer, an instrument validated for clinical use in evaluation of thrombotic diseases and the effectiveness of anti-platelet drugs. The rationale for this trial was: 1) lower requirements for donor's blood to perform the test, 2) availability of a commercial testing instrument, 3) accepted principle of a rotating cone and plate as a source of shear stress, as it is used in rheometers or viscometers. The study design allowed also to test inter-day variability of a single donor blood, to ascertain its impact on the reproducibility of results.

2. Material and methods

A fasting venous blood (4×4.5 mL) was drawn from the same male healthy donor (age 50 years) into sodium citrate tubes (0.5 mL, 0.105M concentration, BD Vacutainer Systems, Franklin Lakes, NY, USA) at one week intervals. A wide gauge needle (12G) was used for blood sampling without any stasis to prevent platelet activation. For the same reason, the first sampling tube was rejected. Measurement of complete blood count was done using Sysmex K-1000 cell counter (Sysmex Co., Mundelein, IL, USA) calibrated each day. Aliquots of blood (0.4 mL) were stored at room temperature and were used for testing within 45 minutes. An additional blood aliquot was sampled at the time of the shear stress to investigate impact of static storage on platelet function. Another aliquot of blood was activated with adenosine diphosphate (ADP, 20 μ M final concentration) for 5 minutes. The control samples were processed in parallel with each replicate of the shear stress experiment. These samples served baseline control for the static conditions and to test platelets capacity to activate. Testing of materials in the shear stress conditions was performed with cone-and-plate(let) analyzer (CPA, Impact-R, DiaMed AG, Switzerland), equipped with an optical microscope module, designed to automatically count number of adherent platelets and their coverage area on the polystyrene (PS) plate (DiaMed Impact-R Test Kit). This procedure is useless for non-transparent materials [4], thus measurements of the platelet count, platelet activation, formation of platelet

aggregates, formation of granulocyte-platelet aggregates, and thrombotic potential of generated plasma microparticles were used instead, to assess biocompatibility of materials. As recommended by the manufacturer, 130 μ L blood volume was used for each shear stress test and the aliquot of the blood was gently mixed for 60 seconds on the rotational wheel (10 revolutions per minute) to prevent sedimentation of blood cells before each of the replicates. PS surface was the original disposable insert well of the Impact-R kit, used for testing of platelets. Polyurethane (PU, Elasthane 55D, DSM PTG, Berkeley, CA, USA) and other materials tested were cut out as disks of 19.5 mm diameter and 2 mm thickness, which fitted the insert well tightly. PU discs were kindly provided by the Foundation for the Development of Cardiosurgery, Zabrze, Poland. Other test discs were provided by Institute of Metallurgy and Materials Sciences, Cracow, Poland. Surface of PU was coated with diamond-like carbon (DLC), titanium (Ti), titanium nitride (TiN), titanium nitride modified with carbon (TiCN), titanium oxide (TiO), silicon modified DLC (SiDLC) and titanium modified DLC (TiDLC). The shear test was applied at shear rate 1800-s (720 revolutions per minute) for 300 seconds, using a disposable teflon conical rotor. Following the shear test, the rotor was carefully removed and blood was immediately sampled from the well to the test tubes, for flow cytometry staining. From the remaining blood (80 μ L) plasma was separated by centrifugation at 2 000 g for 5 min and stored frozen in -70°C for further analysis of thrombotic activity.

2.1. Flow cytometry analysis of the blood. Two sets of tubes were prepared for each tested plate and for the static controls. Expression of platelet activation markers was ascertained using whole blood staining. In brief, 5 μ L aliquots of blood were gently mixed with fluorochrome-conjugated monoclonal antibodies: 5 μ L FITC-PAC-1, 5 μ L PE-CD62P and 4 μ L PerCP-CD61 (all from Becton Dickinson, USA) in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin and 2 mM calcium chloride (final volume 35 μ L). After 10 minutes staining at room temperature, erythrocytes were lysed by addition of 0.5 mL lysing solution (FLS, Becton Dickinson, USA) and platelets were centrifuged (1 000 g, 6 minutes) and resuspended in PBS buffer for further analysis by flow cytometry.

Samples were analyzed using EPICS XL flow cytometer (Beckman Coulter Inc., Brea, CA, USA). Expression of platelet activation markers [5] was measured on CD61 gated objects using PAC-1 antibody for conformational change of glycoprotein IIb/IIIa, and using CD62P for P-selectin. Integrated fluorescence of the activation marker was calculated as a multiplication total of geometric mean fluorescence by percentage of marker-positive objects.

Aggregates of platelets were analyzed after erythrocyte lysis by mixing 25 μ L of blood with 0.4 mL FLS and subsequent fixation by addition 3.5 mL 1% paraformaldehyde in PBS. Cellular material was recovered by centrifugation (1 000 g, 7 minutes) and immunostained (25 μ L aliquots) with 4 μ L PerCP-CD14 and 5 μ L FITC-CD61 or 5 μ L FITC-

CD61 alone for 30 minutes at room temperature. Samples were then washed in PBS, and analyzed by flow cytometry. The percentage of granulocyte-platelet aggregates (leukocytes stained with platelet marker CD61) was calculated using granulocyte forward/side scatter gate and additional monocyte CD14+ gate. Absolute number of platelets was calculated as the number of CD61 positive objects in reference to total granulocytes count. Small and big platelet aggregates were counted using forward/side scatter gates for CD61 positive objects. All other chemicals were obtained from Sigma-Aldrich.

2.2. Measurements of thrombogenic microparticles activity. Thrombogenic potential of blood plasma was measured using Zymuphen MP-activity ELISA kit (Hyphen Biomed, Eragny, France), according to the manufacturer instruction. This assay is based on trapping of phospholipid-rich microparticles derived from cell membranes, using immobilized annexin V, followed by reconstitution of thrombin activity with calibrated clotting factors solution. Proteolytic activity of generated thrombin against the chromogenic substrate strictly correlates with concentration of microparticles present in blood plasma [6].

2.3. Statistical analysis. Statistical analysis was performed using SPSS version 17.0 statistical package (SPSS Inc., Chicago, IL, USA) for personal computers. Data are presented as means \pm SD (standard deviation) and ranges. Statistical significance was assumed for type-I error $p < 0.05$.

3. Results

Seven tests were performed in triplicates, always using two reference plates (PS and PU) and different titanium and carbon layers deposited on the PU plate. The same donor's blood platelet count varied from 139 000 to 265 000 / μ L (in average $211\,571 \pm 51\,081$ / μ L). Thus, under physiological conditions inter-day coefficient of variance for blood platelets in the same subject was 24.1%. Platelet count positively correlated with the number of platelets following the shear stress using PS plate ($R^2 = 0.491$; $p < 0.001$) and to lesser extent with PU shear stress ($R^2 = 0.272$; $p = 0.015$). Thus, donor's blood platelet count could be used as a weight for further analysis.

3.1. Platelet aggregates formation during the shear stress.

Under resting conditions, platelet aggregates count varied from 300 to 7 900 per mm^3 , with average $1\,550 \pm 1\,630$. There was no correlation between platelet aggregates count and initial donor's blood platelet count ($R^2 = 0.171$; n.s.). Shear stress increased count of platelet aggregates in the range from 1 420 to 19 750 per mm^3 on PS (mean $8\,800 \pm 5\,150$) and the range from 5 139 to 28 160 per mm^3 on PU (mean $14\,443 \pm 7\,309$). There was a highly significant positive correlation between small platelet aggregates, involving only few platelets, and total count of aggregates using PS plates ($R^2 = 0.833$; $p < 0.001$), while bigger aggregates did not correlate with total platelets aggregates count at all ($R^2 = 0.026$; n.s.). This observation replicated well for smaller aggregates

using PU plates ($R^2 = 0.764$; $p < 0.001$). Using PU surface, bigger platelets aggregates also correlated with a total platelet aggregates count ($R^2 = 0.218$; $p = 0.033$). Results for platelet aggregates after shear stress using tested materials are shown in Fig. 2.

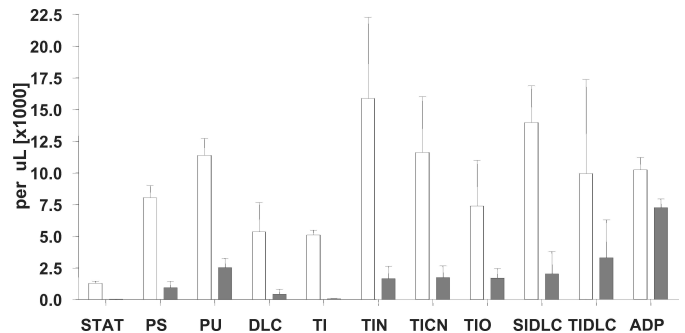


Fig. 2. Platelet aggregates following the shear stress. White columns – small aggregates of a few platelets, grey columns – bigger aggregates > 2 -3 platelets. Controls are: static conditions and ADP activated blood. Materials abbreviations are explained in the Methods section

3.2. Platelets activation following the shear stress. At static conditions of blood storage, percentage of platelets expressing P-selectin was $10.04\% \pm 9.94$. In fully activated platelets, as tested by addition of ADP $20\ \mu\text{M}$ final concentration, the average percentage of platelets expressing this marker was $83.03\% \pm 9.68$. A more informative integrated fluorescence of P-selectin positive platelets, which corresponds to a weighted expression of the marker, pointed out almost twentyfold change following ADP activation from resting 0.68 ± 0.72 fluorescence units to 13.25 ± 4.36 . Using shear stress, PS surface

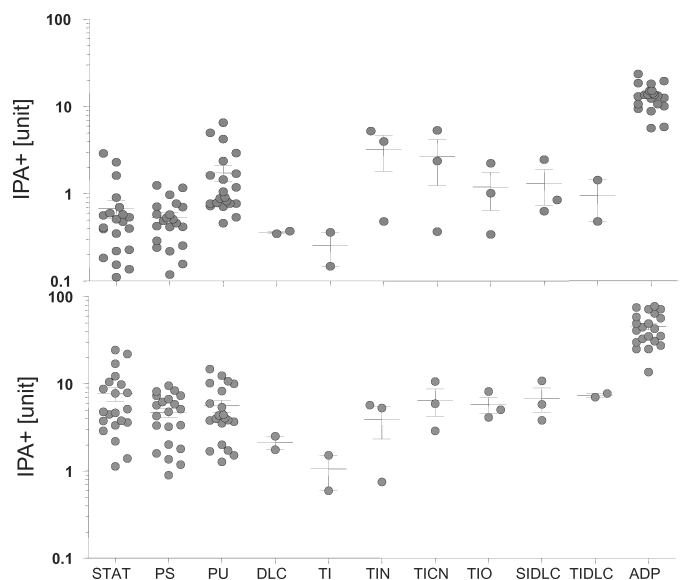


Fig. 3. Platelet activation markers following the shear stress. Upper panel – integrated fluorescence of anti-selectin-P antibody. Lower panel – integrated fluorescence of PAC-1 antibody detecting activated glycoprotein IIb/IIIa. Controls are: static conditions and ADP activated blood. Materials abbreviations are explained in the Methods section

did not significantly increased the percentage of platelets expressing of selectin-P ($7.88\% \pm 4.24$) or the integrated fluorescence of this marker (0.54 ± 0.31 units). This was in contrast with PU surface, which activated platelets on average twofold ($14.25\% \pm 8.52$, and integrated fluorescence to 1.73 ± 1.64). Platelet activation markers from experiments with tested materials are presented in Fig. 3. The other marker for platelet activation, conformational change of glycoprotein IIb/IIIa detected by PAC-1 antibody, revealed similar results. The two markers differ by much higher percentage of platelets expressing PAC-1 antigen.

3.3. Platelet – granulocyte aggregates. In static conditions, in average $17.97\% \pm 8.6$ monocytes had platelets attached to their surface, a phenomenon independent from platelet activation by ADP. This changed rapidly following the shear stress. Using PS surface, the number of monocyte-platelet aggregates decreased to $9.24\% \pm 6.71$. A similar decrease was observed using PU ($8.10\% \pm 1.86$). Tested materials also decreased the percentage of monocyte-platelet aggregates but to a variable extent (Fig. 4). Likewise, neutrophil-platelet aggregates were the most abundant in static conditions ($4.11\% \pm 2.12$). This percentage was not affected by platelet activation with ADP ($5.33\% \pm 3.99$). Following the shear stress the number of aggregates decreased significantly using PS plates ($1.83\% \pm 0.82$) and PU plates ($1.37\% \pm 0.48$). Tested materials also decreased percentage of granulocyte-platelet aggregates (Fig. 4), but the variability was lesser then for monocyte-platelet aggregates.

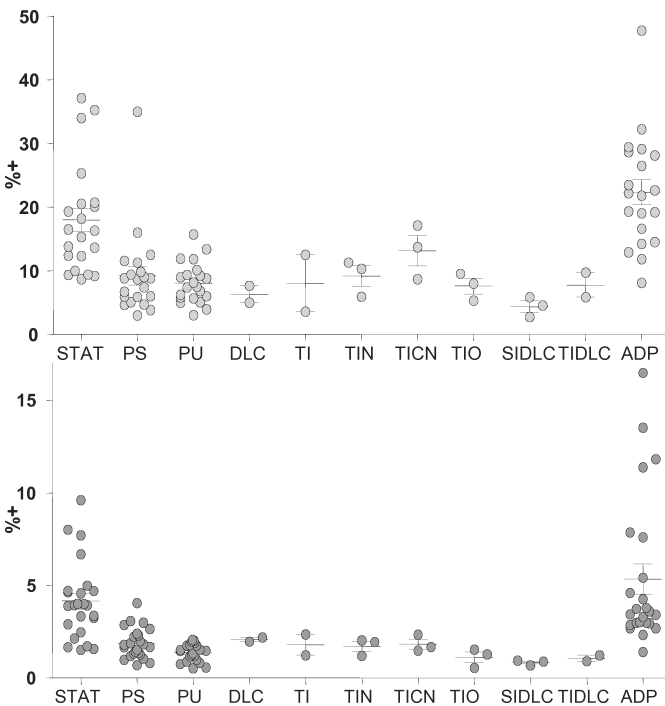


Fig. 4. Granulocyte-platelet aggregates following the shear stress, as a percentage of total granulocytes. Upper panel: monocyte-platelet aggregates. Lower panel: neutrophil-platelet aggregates. Controls are: static conditions and ADP activated blood. Materials abbreviations are explained in the Methods section

3.4. Plasma thrombotic potential resulting from the formation of platelet microparticles. In the static conditions, concentration of blood plasma microparticles was the lowest and averaged 8.6 ± 7.45 nM. This was not increased by ADP activation of the blood, probably due to a rapid aggregation of platelets in excess of ADP. The shear stress conditions always led to the increase in concentration of microparticles. Using PS plates, the increase was almost threefold, to 18.48 ± 12.65 nM and was highly significant ($p < 0.01$). Similar result were obtained using PU plates (19.32 ± 9.61 nM; $p < 0.01$). All tested materials also increased plasma concentration of microparticles (Fig. 5). There was no correlation between the number of platelet aggregates and plasma concentration of microparticles in static or dynamic conditions. However, following the shear stress using PS plates, concentration of microparticles moderately correlated positively with percentage of platelets expressing P-selectin ($R^2 = 0.194$; $p < 0.05$) and highly significantly with P-selectin integrated fluorescence ($R^2 = 0.567$; $p < 0.01$). Using PU plates, this correlation was even more evident (% of P-selectin positive platelets; $R^2 = 0.390$; $p = 0.002$, and P-selectin integrated fluorescence $R^2 = 0.374$; $p = 0.003$). Similarly significant results were observed, when expression of PAC-1 antigen was used as a marker for platelet activation.

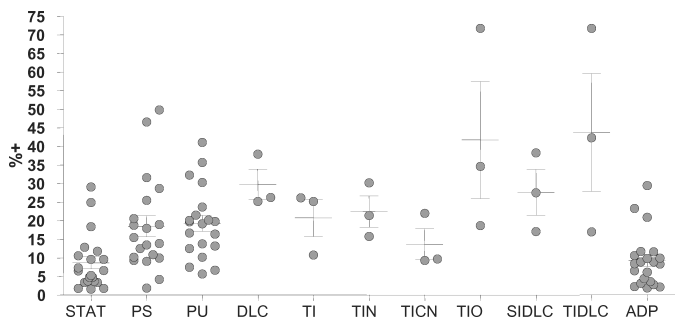


Fig. 5. Microparticles thrombotic activity of blood plasma following the shear stress. Controls are: static conditions and ADP activated blood. Materials abbreviations are explained in the Methods section

3.5. Ranking hemocompatibility of tested materials. A relative rank for hemocompatibility of tested materials was constructed using 2-dimensional plots, where percentage of platelets remaining after the shear stress was plotted against: platelets aggregates, platelet-monocyte aggregates, platelet percentage positive for activation marker P-selectin and concentration of microparticles in blood plasma (Fig. 6). The lower right corner of these scatterplots theoretically represent the most favorable properties of the test material, i.e. no consumption of platelets and the lowest value of the parameter for adverse platelet function. It is noteworthy, that some materials, e.g. TiO performed the worst using either of the parameters, while some other gave results which differed diametrically, for example TiN and TiCN. These two materials substantially activated platelet markers but had the lowest generation of thrombotic activity related to microparticles.

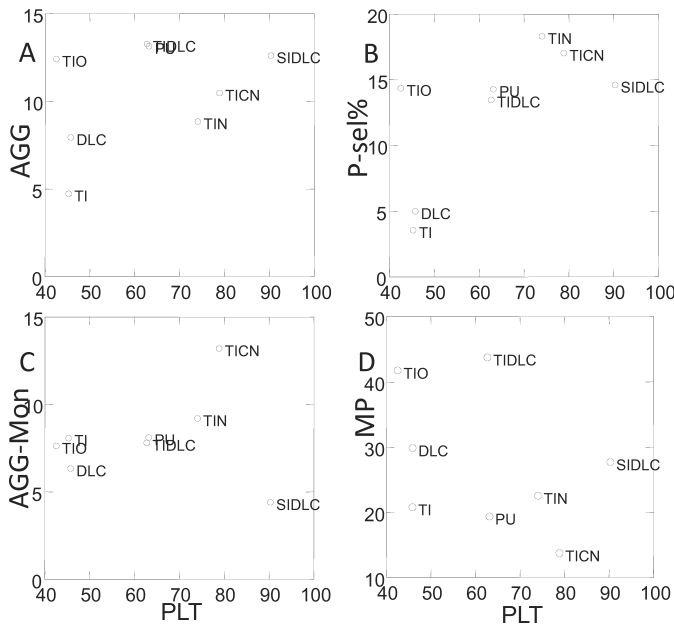


Fig. 6. Plots ranking hemobiocompatibility of tested materials. Panel A: percentage of platelet aggregates (AGG); Panel B: percentage of P-selectin positive platelets (P-sel%); Panel C: percentage of platelet-monocyte aggregates (AGG-Mon); Panel D: concentration of microparticles thrombogenic activity (MP). All values are plotted against the same ordinal of percentage of platelets remained after the shear stress (PLT). Materials abbreviations are explained in the Methods section

4. Discussion

In vitro testing for hemobiocompatibility remains a challenge because the background of interactions between blood and foreign material is not fully explained [7]. It is generally acknowledged that initial and rapid adsorption of plasma proteins can establish further biocompatible properties of the material [8, 9]. The process can be quite selective, as evidenced for PS surface, which covers with large proteins, including von Willebrand factor, a major docking molecule for platelet. From the technical point, quality of the material surface is also very important. During blood flow, any rough surface, its protuberances or cavities, can result in non-laminar flow, and precipitate cells activation and adhesion. Physiological endothelium has very smooth surface, because the cells are flattened to much less than 1 μm thickness. The major limitation of *in vitro* tests for hemocompatibility is due to a short time of the experiment. Blood is a live tissue and viability of platelets deteriorate with storage time longer than 60 minutes along with their spontaneous activation. Moreover, conventional test chambers with laminar flow require volumes of blood which frequently exceed 100 ml during 5 minutes experiment. Inter-individual and inter-day variation of peripheral blood morphology parameters is high and in our observation done on carefully selected donor exceeded 24%. Drugs affecting platelet functions, like non-steroidal anti-inflammatory drugs (e.g. aspirin) readily available over the counter, also have to be taken into consideration. With all these factors contributing to the high variation in the experiments for test-

ing hemocompatibility, we decided to simplify *in vitro* setup of the test by using a well established rotating cone and plate viscometer. ISO guidelines for testing hemocompatibility insist on the usage of standard methods [1]. In line with this, we adopted a commercial instrument, marketed for clinical diagnostics of platelet dysfunction, cone-and-plate(let) analyzer [10]. We also selected conventional biomarkers used in hemocompatibility tests, i.e. platelet destruction, platelet aggregates, granulocyte-platelet aggregates and platelet activation markers. With all of these we demonstrated feasibility to perform the tests. The major advantage of the setup was a low blood volume required for the test and the possibility to study several materials in parallel. A major limitation of this study was 1) a low statistical power due to the insufficient number of replicates of the materials studied, except PS and PU, 2) the fact that the study was not controlled for the mechanical quality of the surface (except PS), as was demonstrated later during a microscopic analysis of the plates. Some of tested discs had surface defects in the form of corrugations, or cracks in the layered materials, 3) microscopic analysis of adherent cells or protein deposits was not performed during the study. Nevertheless, some valid conclusions can be drawn from the current study. Among the studied parameters for hemocompatibility, total number of platelet aggregates should be split into small and bigger aggregates. The smaller aggregates comprising only few platelets adhering together probably mark for the mechanical quality of the surface and result from a moderate activation of platelets. Small aggregates always increase following the shear stress and the increase can be higher using rough surfaces. Bigger aggregates were observed only with some plates tested and can indicate two opposite processes. Increased number of bigger aggregates is observed with higher activation of platelets, but adherence of these aggregates to the material surface makes this marker incredulous. Similar comments apply to the markers of platelet activation. Lack of adherence of activated platelet to the test surface can explain contradictory results of TiN and TiCN tests. These materials showed a quite high expression of P-selectin and PAC-1 activation markers, but microparticles activity in blood plasma was among the lowest. Microparticles activity of blood plasma, which in our *in vitro* tests has reflected fragmentation of platelets and release of cell membrane vesicles during exocytosis seems to be a novel finding of this study. Microparticles impose thrombotic burden to circulating blood [6] and were recently found to correlate with atherosclerosis and risk for major adverse cardiovascular events, like heart infarct or stroke [11, 12]. Though generation of platelet microparticles was noted using flow cytometry almost two decades ago [13], using *in vitro* models of whole blood activation, only recently thrombotic function has become measurable with modern ELISA assays [6]. This parameter, correlating with a clinical prognosis for the patient, seems of the highest importance in testing hemobiocompatibility of biomaterials. It is also one of the markers, which can be used in monitoring performance of cardiovascular prostheses, since it can be measured *in vivo*.

4.1. Summary. Using a simplified experimental setup, based on inexpensive commercial cone-and-plate analyzer, we managed to reproduce the same events, which seems limiting usage of artificial materials in contact with blood. The advantage of the proposed method is low volume of donor's blood required for testing and higher throughput of the method, enabling parallel testing of materials. This method inherits the same drawbacks, as other experimental setups for hemobiocompatibility testing. Due to a high physiological variation in blood morphology, mainly platelet count, credible results can be obtained only using 8–10 replicates of the test, as was demonstrated by other authors [3, 4]. Moreover, the test is also very sensitive to the imperfect smoothness of the surface. This limitation can be evaded using a high quality test plates. Ranking of PU coatings, despite insufficient number of tested plates, revealed that TiN, TiCN and possibly SiDLC have the best hemobiocompatibility profiles and should be selected for more extensive tests.

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